UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

Social memory in zebrafish: behavioral assessment and the role of Brain-Derived Neurotrophic Factor (BDNF)

Natália da Costa Madeira

Orientador: Professor Doutor Rui Filipe Nunes Pais de Oliveira

Co-Orientador: Professora Doutora Maria José de Oliveira Diógenes

Master Degree in Neurosciences

2015

Todas as afirmações efectua das no presente documento são da exclusiva responsabilidade do seu autor, não cabendo qualquer responsabilidade à Faculdade de Medicina de Lisboa pelos conteúdos nele apresentados.

A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 23 de Julho de 2015

AGRADECIMENTOS

A realização deste trabalho não teria sido possível sem a colaboração, compreensão e ajuda de inúmeras pessoas. Após este período de intensa aprendizagem científica e pessoal, quero deixar o meu profundo agradecimento a todos os que me apoiaram ao longo deste percurso académico:

Ao Instituto Gulbenkian de Ciência, por me ter acolhido durante o decurso da tese e pelas aprendizagens multidisciplinares que adquiri;

Ao Professor Doutor Rui Oliveira, pela oportunidade de desenvolver o meu trabalho no seu grupo de investigação e por todo o conhecimento que me transmitiu;

À Professora Doutora Maria José Diógenes, por me ter aceite a co-supervisão deste trabalho;

À Magda Teles, por todo o apoio e dedicação durante este percurso; por todas as técnicas e conhecimento que me transmitiu. Principalmente por toda a amizade que me ofecereu.

À Júlia Pinho, pelo apoio árduo e incansável de uma verdadeira amiga. Sem dúvida que foste um elemento chave para o sucesso deste trabalho.

Aos membros do IBBG (Ana Faustino, Rodrigo Abreu, João Lopes, Ana Rita Nunes, André Monteiro, Ana Sofia Félix, António Roleira e Ana Cruz), pelo apoio demonstrado ao longo destes meses;

À minha mãe, por me ter fascinado desde a infância pelo maravilhoso mundo do conhecimento. Pela maravilhosa forma como me fez ver a natureza, as pessoas os animais e o mundo.

À minha familía, pelo apoio incondicional;

Ao meu namorado, por todo o amor e companheirismo;

À Mariana Marques, pela enorme amizade. Pelo interesse e satisfação que demonstrava quando falávamos do meu projecto e por todas as dicas. És e continuarás a ser uma das grandes marcas no meu percurso académico;

À Carolina Toste, por todo o apoio, amizade e carinho que demonstrou ao longo do mestrado. Pela sua enorme capacidade crítica e pelo seu infinito conhecimento que nos inspira para continuar neste caminho.

À Rita Belo, pela enorme amizade, carinho e dedicação que demonstrou a longo destes anos.

Ao Luís Ceríaco, pela amizade e carinho. Por me teres conduzido desde cedo ao mundo da investigação e me teres proporcionado muito conhecimento.

Ao Professor Doutor Paulo de Oliveira, por me ter ajudado e incentivado a descobrir o mundo das Neurociências e por toda a confiança depositada em mim.

INDEX

LIST OF TABLES ..... **xiii**

LIST OF FIGURES ..... **xiv**

ABBREVIATIONS ..... **xvii**

RESUMO ..... **xxi**

ABSTRACT ..... **xxv**

CHAPTER 1 – INTRODUCTION **1**

1. Behavioral flexibility in social context 3

2. Neuronal plasticity 5

2.1. Molecular mechanisms underlying neuronal plasticity and its influence on memory formation 6

2.2. Brain-derived neurotrophic factor 9

2.2.1. BDNF and memory processing 11

3. Social brain 12

3.1 Hippocampus 14

4. Social recognition memory 16

4.1. Neurobiological basis of social memory 18

4.2. Zebrafish as a model for study social memory 21

CHAPTER 2 – OBJETIVES **25**

CHAPTER 3 – METHODS **27**

1. Time dependent expression of *c-fos* and *bdnf* 29

Animals housing 31

Experimental procedure 31

Sampling 32

Western-blotting 33

Stripping and re-probing membranes 33

RNA isolation, cDNA synthesis and real-time quantitative PCR amplification 34

qPCR data processing 35

Statistics analysis 35

2. Assessment of long-term memory for social and non-social recognition in zebrafish 36

Animals housing 36

Animal tagging 37

Experimental tests 43

Brain tissue sampling 44

RNA isolation, cDNA synthesis and real-time quantitative PCR amplification 45

Statistics analysis 45

CHAPTER 4 – RESULTS 47

Effects of Kainic acid on *c-fos* and *bdnf* gene expression in the telencephalon 49

Effects of Kainic acid on C-FOS and BDNF protein levels in the telencephalon 52

Object recognition 56

Effects of different sensory modalities in social recognition using the social discrimination test 57

Test of social recognition using the habituation-dishabituation paradigm 59

Sociability and memory performance 61

Effects of social memory on *bdnf* gene expression in the hippocampus and amygdala 62

CHAPTER 5 – DISCUSSION 65

CHAPTER 6 – CONCLUSIONS 73

REFERENCES 77

LIST OF TABLES

TABLE 1|PRIMER SEQUENCES, ANNEALING TEMPERATURE AND EFFICIENCY. ALL PRIMERS WERE COMMERCIALLY SYNTHESIZED (SIGMA-ALDRICH, GERMANY). 35

TABLE 2| ANOVA ANALYSES FOR C-FOS AND BDNF GENE EXPRESSION AFTER A TREATMENT WITH KAINIC ACID (KA) AND SALINE SOLUTION (CT) AT DIFFERENT SAMPLING TIMEPOINTS (0H, 0.5H, 1H, 2H, 4H, 8H, 24H AND 48H}. MAIN EFFECTS AND RESULTS FROM PLANNED COMPARISONS ARE PRESENT IN THIS TABLE. ABBREVIATIONS: B, BASELINE; CT, CONTROL GROUP; KA, KAINIC ACID GROUP. *P<0.005. 51

TABLE 3 | ANOVA ANALYSES FOR C-FOS GENE EXPRESSION AFTER A TREATMENT WITH KAINIC ACID (KA) OR SALINE SOLUTION (CT) AT DIFFERENT SAMPLING TIMEPOINTS (0H, 0.5H, 1H, 2H, 4H, 8H, 24H AND 48H). MAIN EFFECTS AND RESULTS FROM PLANNED COMPARISONS ARE PRESENT IN THIS TABLE. ABBREVIATIONS: B, BASELINE; CT, CONTROL GROUP; KA, KAINIC ACID GROUP. *P<0.005. 54

TABLE 4 | RESULTS DETERMINED BY ANOVA FOLLOWED BY TUKEY'S POST-HOC TEST...... 62

LIST OF FIGURES

FIGURE 1 |MODEL OF SYNAPTIC TRANSMISSION AT EXCITATORY SYNAPSES (CITRI AND MALENKA, 2008) 7

FIGURE 2 | MODEL OF BDNF-P75NTR AND BDNF-TRKB INTRACELLULAR SIGNALING PATHWAYS (CUNHA ET AL., 2010) 10

FIGURE 3| SCHEMATIC REPRESENTATION OF EXPERIMENTAL PROCEDURE. AFTER ADMINISTRATION OF KA OR SALINE SOLUTION, ANIMALS WERE SACRIFICED AT DIFFERENT TIMEPOINTS. 32

FIGURE 4|BRAIN AREAS COLLECTED. A) MID SAGITTAL CUT; RIGHT SIDE FOR QPCR AND LEFT SIDE FOR WESTERN-BLOTTING; B) IN EACH HALF PART OF THE BRAIN, FIVE MACROAREAS WERE COLLECTED. 32

FIGURE 5 |TOP VIEW OF EXPERIMENTAL SET-UP. THE GREY DOTTED LINES REPRESENT REMOVABLE PARTITIONS AND THE YELLOW SQUARES REPRESENT THE LOCALIZATION OF DEMONSTRATORS' FISH..... 37

FIGURE 6 | SCHEMATIC REPRESENTATION OF BEHAVIORAL PROCEDURE 38

FIGURE 7 | DIAGRAMS OF THE RECOGNITION MEMORY TASKS. A) OBJECT DISCRIMINATION TEST; B) SOCIAL DISCRIMINATION TEST AND C) SOCIABILITY TEST. 42

FIGURE 8 |TEMPORAL CHANGES IN IEG'S EXPRESSION AFTER A TREATMENT WITH KAINIC ACID. ANIMALS WERE INJECTED WITH EITHER SALINE OR KAINIC ACID SOLUTION AND SACRIFICED IMMEDIATELY, 30 MIN., 1HR, 2HR, 4HR, 8HR, 24HR OR 48HR AFTER INJECTIONS. THE TIME COURSE, AFTER EITHER SALINE OR KA ADMINISTRATION, FOR INCREASES IN RELATIVE LEVELS OF MRNA IN TELENCEPHALON ARE SHOWN FOR C-FOS (A) AND BDNF (B). ACUTE INTRAPERITONEAL KAINIC ACID (0.5MG/KG) INCREASES (A) C-FOS EXPRESSION 30 MINUTES AFTER TREATMENT; (B) BDNF EXPRESSION 0 MINUTES AFTER SALINE SOLUTION ADMINISTRATION. THE RESULTS ARE THE MEAN±SEM OF SEVENTEEN DIFFERENT EXPERIMENTS PERFORMED IN INDEPENDENT ANIMALS. *P≤0.05; ***P≤0.001. 49

FIGURE 9 | TEMPORAL CHANGES IN C-FOS PROTEIN LEVELS AFTER A TREATMENT WITH KAINIC ACID. ANIMALS WERE INJECTED WITH EITHER SALINE OR KAINIC ACID SOLUTION AND SACRIFICED IMMEDIATELY, 30 MIN., 1HR, 2HR, 4HR, 8HR, 24HR OR 48HR AFTER INJECTIONS. THE TIME COURSE, AFTER EITHER SALINE OR KA ADMINISTRATION, FOR INCREASES IN PROTEIN LEVELS IN TELENCEPHALON ARE SHOWN FOR C-FOS. ACUTE

intraperitoneal kainic acid (0.5mg/Kg) does not induces an increase in protein levels. The results are the mean±SEM of seventeen different experiments performed in independent animals. *p≤0.05; **p≤0.01; ***p≤0.001 52

Figure 10 | Temporal changes in BDNF protein levels after treatment with kainic acid: A) pro-BDNF immunoreactivity; B) mature-BDNF immunoreactivity. Animals were injected with either saline or kainic acid solution and sacrificed immediately, 30 min., 1hr, 2hr, 4hr, 8hr, 24hr or 48hr after injections. The time course, after either saline or KA administration, for increases in protein levels in telencephalon are shown for BDNF. Acute intraperitoneal kainic acid (0.5mg/Kg) induces an increase in pro-BDNF levels 0hrs, 1hrs, 24hrs and 48hrs after administration; following an increase in mature-BDNF levels at 0hrs, 1hrs, 2hrs, 4hrs, 8hrs, 24hrs and 48hrs. The results are the mean±SEM of seventeen different experiments performed in independent animals. *p≤0.05; **p≤0.01; ***p≤0.001 53

Figure 11| Object recognition. The time spent in each chamber were used to investigate object recognition. On day 1, animals were freely to explore two naive objects different in shape but with the same volume and color. 24hr later, individuals were faced with a familiar and a non-familiar object. Focal fish cannot discriminate between a familiar and a non-familiar object. A) Preference score and B) exploration time (%) *p≤0.05 56

Figure 12 | Social memory assessment for olfactory (A), visual (B) and integration of both (C) stimuli. The time spent in each chamber were used to investigate social recognition, based on different types of sensorial modalities. On day 1, animals were freely to explore two naive conspecifics. 24hr later, individuals were faced with a familiar and a non-familiar conspecifics. A) Focal fish cannot discriminate familiar from non-familiar conspecifics only based on olfactory interaction; B) Focal fish discriminate familiar from non-familiar conspecifics only based on visual interaction; C) Focal fish discriminate familiar from non-familiar conspecifics based on visual and olfactory interactions. $^{+}$p<0.06; *p≤0.05 57

Figure 13 |Stimulus exploration (%) for social discrimination memory using olfactory (A), visual (B) and multimodal (C) cues. On the first day animals explore actively the conspecifics. On the second day individuals reduce significantly the exploration time. 58

Figure 14 | Social memory assessment using a habituation-dishabituation paradigm. The time spent in each chamber were used to investigate social recognition. On day 1, animals were freely to explore one naive conspecific. 24hr later, individuals were faced with a novel (A) or a familiar conspecific (B) 59

FIGURE 15 | CORRELATION BETWEEN SOCIAL MEMORY PERFORMANCE AND SOCIABILITY. 600

FIGURE 16 | BDNF GENE EXPRESSION CHANGES AFTER MEMORY TESTS. *P≤0.05..... 611

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CaMKII	calcium/calmodulin dependent kinase II
cAMP	cyclic adenosine monophosphate
CNS	Central nervous system
CREB	cAMP response element-binding protein
Dl	Lateral zone of dorsal telencephalic area
Dm	Medial zone of dorsal telencephalic area
EGR-1	Early growth response protein 1
ELF1-a	Elongation factor 1
FFA	Fusiform face area
HEPES	N-(2-hydroxyethyl)-1-piperazine-N'-(2-ethanesulfonic acid)
IEG	Immediate-early gene
KO	Knockout
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinases
MRS	Mesolimbic reward system
NMDA	N-methyl-D-aspartate
OT	Oxytocin
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase

PKA	Protein kinase a
PLCγ	Phospholipase C gamma
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SBN	Social brain network
SEM	Standard error of the mean
TrkB	Tropomyosin-related kinase B

Resumo

Os comportamentos sociais requerem uma elevada flexibilidade comportamental, nos quais conhecimentos adquiridos *a priori* são fulcrais para a adaptação a novas situações. No reino animal, as espécies diferem na sua capacidade social: espécies sociais (gregárias) formam grupos sociais coesos e demonstram relações afiliativas entre os vários membros do grupo; contrariamente a espécies associais. Desta forma, espécies gregárias interagem diariamente com outras, onde a capacidade de armazenar e recordar informações se torna claramente importante. Na natureza, a capacidade de recordar informações relativas ao meio ambiente, relembrar a localização de estímulos recompensatórios e a identificação de indivíduos familiares, assume extrema importância do ponto de vista ecológico. O conhecimento que os animais adquirem acerca dos seus conspecíficos e a forma como constroem o conhecimento do mundo social circundante envolve, invariavelmente, a capacidade de categorizar os conspecíficos (por exemplo: idade, género, hierarquia, entre outros). A capacidade de distinguir indivíduos e armazenar essa informação durante longos períodos de tempo é uma vantagem social que facilita interacções posteriores e é essencial para os comportamentos sociais. O reconhecimento social tem como base características sensoriais multimodais que, conjuntamente, permitem o reconhecimento de indivíduos. Em condições laboratoriais, a memória social pode ser estudada recorrendo a dois paradigmas amplamente utilizados em roedores: teste de discriminação social binária e teste de habituação-desabituação. Ambos os paradigmas baseiam-se em alterações comportamentais espontâneas na exploração dos indivíduos, quando re-expostos a indivíduos familiares.

O presente trabalho avalia a performance do peixe-zebra nas duas variantes do teste de memória social e num teste de memória asocial. Desta forma, 48 animais foram submetidos a uma série de seis paradigmas comportamentais. Cada indivíduo realizou os seguintes testes experimentais: 1) reconhecimento de objectos (O); reconhecimento social: 2) químico (C); 3) visual (V); 4) visual e químico (V+C); Teste de habituação-desabituação: 5) exploração de indivíduos novos (N+N); 6) exploração de individuos novos e familiares (N+F). Os animais foram inicialmente identificados (código de cores) para possibilitar a sua análise individual em cada teste. Ao longo das experiências os animais encontraram-se agrupados sem manterem contacto com os indivíduos "estímulo". Os vídeos recolhidos durante aos experiências foram analisados utilizando um programa de video-tracking (Ethovision®). Para cada indivíduo foi calculado um score de

preferência – relaciona o tempo de investigação de um estímulo com o tempo de investigação de ambos – e a taxa de exploração – relaciona a percentagem de tempo de exploração de ambos os estímulos tendo em conta o tempo total do teste. Os resultados comportamentais revelaram que: i) o peixe-zebra não demonstrou preferência por nenhum dos objectos apresentados 24 horas após o primeiro teste; ii) os indivíduos apresentaram preferência por indivíduos não-familiares quando a interação envolveu estímulos visuais e quiímicos; iii) apresentam preferência por indivíduos familiares quando a interacção tem por base estímulos somente visuais; iv) não demonstram preferência por nenhum dos indivíduos quando dispunham somente de estímulos químicos; v) o paradigma comportamental de habituação-desabituação não demonstrou preferência por animais novos ou familiares, 24h após o primeiro teste. Os resultados exploratórios revelaram que durante o primeiro dia de experiência, os indivíduos apresentaram taxas de exploração acima dos 50%, demonstrando a tendência natural do peixe-zebra em explorar novos estímulos. No entanto, 24 horas após o primeiro teste, os animais reduziram significativamente os níveis de exploração; sugerindo habituação ao teste do segundo dia.

Inúmeros estudos têm sugerido a amigdala como a área cerebral onde a memória social está alocada, sugerindo os neuropeptidos (oxitocina e vasopressina) como reguladores deste tipo de memória. No entanto, até ao momento, nenhum estudo demonstrou o envolvimento do BDNF na memória social. Desde que o brain-derived neurotrophic factor (BDNF) provou estar envolvido em mecanismos de plasticidade sináptica, o seu papel em diversos mecanismos de memória e aprendizagem foi amplamente demonstrado. No processamento da memória, o BDNF pode actuar a vários níveis moleculares: regula canais iónicos como o canal de Na^+ e canal de K^+; modula receptores glutamatérgicos (NMDA e AMPA) e afecta a síntese proteica. Desta forma, o presente trabalho pretendeu avaliar o papel do BDNF na memória social. Os paradigmas comportamentais foram inicialmente balanceados, o que significa que cada grupo de animais terminaria o conjunto dos testes em paradigmas comportamentais diferentes. Desta forma, 2 horas após o término dos testes comportamentais os animais foram sacrificados. As áreas homólogas ao hipocampo (Dl) e à amigdala (Dm) foram extraídas e posteriormente analisados por qPCR. A análise da expressão genética demonstrou que os diferentes testes comportamentais produziam diferenças nos níveis de expressão do *bdnf*. Na amigdala, não se verificaram diferenças signicativas nos níveis de expressão do *bdnf*. No entanto, ao nível do hipocampo verificou-se que os indivíduos que realizaram o teste de memória social com base em estímulos químicos (C) possuia níveis de

expressão de *bdnf* significativamente menores aos testes de memória social com base em estímulos visuais ou visuais (V) e químicos (V+C).

Por outro lado, foi possível correlacionar o teste de memória social com a sociabilidade. Desta forma, foi possível estabelecer uma correlação significativa entre os indivíduos mais sociais e os que apresentam maior capacidade de reconhecimento na realização do teste de memória social.

O último objectivo do trabalho pretendia perceber a forma como os genes de expressão imediata (immediate early genes – IEG's) poderão funcionar como marcadores de resposta neural. Desta forma, foram analisados 128 animais submetidos a um tratamento com ácido kaínico para posterior análise do *c-fos* e *bdnf*. Os indivíduos foram injectados intraperitonialmente com 0.5mg/Kg de ácido kainico (grupo tratamento) ou solução salina (grupo controlo) e, posteriormente, sacrificados a diferentes tempos de amostragem (imediatamente após a injecção; 0 minutos; 30 minutos; 1 hora; 2 horas; 4 horas; 8 horas; 24 horas; 48 horas após a injecção). Posteriormente, os cérebros foram recolhidos e dissecaram-se as 5 principais macroareas: telencéfalo; diencéfalo; tecto óptico; cerebelo e tronco cerebral. Em cada individuo, foi realizado um corte sagital entre os dois hemisférios cerebrais; metade do cérebro foi alocado para a análise da expressão génica (qPCR) enquanto o restante foi utilizada para avaliar os níveis proteicos (Western-blot). Os resultados revelaram que, após um insulto externo, ocorre um aumento significativo nos níveis de expressão do *c-fos* após 30 minutos. Subsequentemente, regista-se uma diminuição abrupta da expressão de *c-fos* que se mantém ao longo de 48 horas. No entanto, os resultados da análise proteica não revelaram diferenças significativas nos níveis de proteinas do C-FOS ao longo do tempo e entre os tratamentos (controlo vs tratamento). Relativamente ao *bdnf*, verificou-se um aumento significativo nos níveis expressão 0 minutos após a administração de uma substância salina, que se mantém no mínimo até 24 horas. 48 horas após a administração, ambos os tratamentos (ácido kaínico e solução salina) diminuem significativamente os níveis de expressão do *bdnf*. A nível proteico, avaliou-se as duas isoformas do BDNF – pro-BDNF e mature-BDNF. Os níveis da isoforma pro-BDNF sofrem um aumento significativo 2 e 8 horas após a administração da solução salina; e 0, 1, 4, 24 e 48 horas após a administração do ácido kaínico. A isoforma mature-BDNF revelou um aumento significativo 0 e 30 minutos após a administração da solução salina; e 0 minutos, 1h, 2h, 4h, 8h, 24h e 48h após a administração do ácido kaínico.

Os resultados obtidos pretendem demonstrar a utilidade do peixe-zebra no estudo da memória social, através da validação de paradigmas comportamentais para esse efeito. Por outro lado, demonstrámos que o hipocampo tem um papel neste tipo de memória que é dependente da origem sensorial dos estímulos utilizados.

Palavras-chave: memória social; peixe-zebra; BDNF; hipocampo

Abstract

The ability of animals to gather information about their social and physical environment is essential for their ecological function. Animals are often organize conspecifics into categories (e.g. sex, age, hierarchical status). This social organization is underpinned by social recognition. Individuals use an assortment of different cues to obtain information about their environment and to recognize the individuals that they encounter. The present study evaluated the influence of 3 different sensory cues on social recognition: visual-only; olfactory-only and visual + olfactory. We used two different paradigms to assess social recognition memory – social discrimination paradigm and a habituation-dishabituation paradigm – both adapted from mouse studies. We also explored an asocial task – novel object test. A series of six experiments were performed by each individual. Subsequently, *bdnf* expression levels were evaluated in hippocampus and amygdala.

The behavioral results show that zebrafish: i) did not demonstrate preference for any of the objects presented 24h after the initial test; ii) preferentially associated with conspecifics that are novel, when using both chemical and visual cues; iii) exhibit preference for familiar conspecifics when only visually cues are accessible; iv) did not show preference between two individuals, when only chemical cues were available; v) failed to demonstrate social recognition memory using the habituation-dishabituation paradigm; vi) are highly inquisitive animals. The genetic expression analysis demonstrates no differences in *bdnf* expression levels in the amygdala. However, in the hippocampus, low-levels of *bdnf* were present when animals performed a discrimination paradigm based only on olfactory cues; in contrast with individuals that performed the same behavioral paradigm based on visual and visual + chemical cues.). Here we propose that the high levels of BDNF observed in the Dl could affect LTP and consequently the production and secretion of OT in the Dm. Our findings present a new possibility for the role of neural connections between the Dl and Dm regions, mediated by BDNF, with significant impact on social memory.

We also evaluated the use of immediate early genes (*c-fos* and *bdnf*) as neural response markers in the zebrafish telencephalon. We analyzed animals that were submitted to a kainic acid treatment. To study the temporal response of IEG's (*c-fos* and *bdnf*) to the treatment, an analysis of gene expression (qPCR) and protein levels (Western-blot) was performed. Animals were given intraperitoneal injections (0.5mg/Kg) of saline solution (control group) or kainic acid (treatment group) and sacrificed at different sampling time points (immediately after, 0min., 30min., 1h, 2h,

4h, 8h, 24h and 48h). The results show that *c-fos* suffered an up-regulation 30 min. after treatment; followed by an abrupt decrease in *c-fos* expression levels. However, protein levels did not show significant differences in C-FOS protein. Regarding bdnf: an up-regulation was observed 0min. after saline solution administration; 48h after administration of either KA or saline solution, a decrease in *bdnf* expression levels was observed. At the protein level both pro-BDNF and mature-BDNF levels were analyzed. Pro-BDNF levels increase 2h and 8h after saline solution administration; and 0min., 1h, 4h, 24h and 48h after KA administration. In contrast, mature-BDNF levels increase 0min. and 30min. after saline solution administration; and 0min., 1h, 2h, 4h, 8h, 24h and 48h after KA administration.

The present work demonstrates the usefulness of zebrafish in studying social memory, by the validation of paradigms to that effect. This work also suggests that the hippocampus possesses a role in this type of memory, depending on the origin of the cues employed.

Key-words: social memory; zebrafish; BDNF; hippocampus

CHAPTER 1

Introduction

1. BEHAVIORAL FLEXIBILITY IN SOCIAL CONTEXT

Everyday behaviors require a high degree of flexibility, in which prior knowledge is used to adapt to new situations. Understanding the processes and mechanisms by which animals act, learn, remember and use this information to navigate their daily lives, is currently one of the goals in the field of neurosciences. Thus, studying how the brain can produce complex behaviors and cognitive states, and how it can be influenced by social experience is one of key themes in cognitive neuroscience.

Behavior interfaces an animal characteristics – genetic, physiology and personality traits – and its environment. Animals must act to select suitable habitats; to maintain homeostasis; to avoid predation; to find and select mates; to rear their offspring successfully and to manage their social relationships with conspecifics (Kappeler et al., 2013). However, in unpredictable environments animals require an extraordinary ability in modifying behaviors, also known as behavioral flexibility. Across animals, there is a remarkable diversity in naturally occurring behavioral phenotypes. Adaptive behavioral solutions to recurrent, unpredictable environments and social problems should therefore be favored by selection, resulting in robust, species-specific behavior patterns (Duckworth, 2009; Sih et al., 2010). Such flexibility is thought to be supported in part by memory integration.

Social interactions promote changes in morphology, and/or physiology of interacting individuals. There is a large number of gregarious species in the animal kingdom. Contrary to solitary species – with high levels of territorial defenses that lead to the active exclusion of conspecifics – individuals of social species establish relationships with each other. Social species have the ability to adjust their behavior according to previous social experiences and social contexts (Oliveira, 2009). This behavioral flexibility, caused by changes in social environments, results in an optimization of relationships between organisms, known as “social plasticity”. Social plasticity is a process that can be divided into different phases: 1st) animals collect relevant cues from the social environment; 2nd) they evaluate the salience and valence of social stimulus (appraisal mechanism); 3rd) mechanisms of cognitive appraisal result in different forms of neuronal plasticity: short-term changes (activation of proteins that act as transcription factors (CREB); neuronal activity-dependent transcription factors activate IEG’s that can encode other transcription factors (c-fos and egr-1) or synaptic proteins (Arc and Homer1a); and transcription of

miRNAs that regulate translation of synaptic proteins) or long-term changes (epigenetic modifications of genes involved in social behavior (e.g. *bdnf*)); 4th) temporal and spatial changes in gene regulation in the social brain network (SBN); 5th) production of hormones or neuromodulators that can change the strength of connectivity between the nodes of SBN (Oliveira, 2012).

In the specific case of recognition, the identification of conspecifics is essential for the processing of social information. This implies the precise regulation of specific brain mechanisms associated with the recognition, and interpretation of several aspects of social information.

2. NEURONAL PLASTICITY

The nervous system has the ability to adapt to the environment and to improve its performance with experience. Most learning processes result in long-lasting behavioral changes, but even simple reflexes can be modified transiently. The fact that behavior is learned raises an interesting question: how is behavior modified if the nervous system is wired so precisely? How can changes in neural control of behavior occur when connections between the signaling units, the neurons, are set during early development? The proposal that has proven farsighted is the (neuro)plasticity hypothesis.

Neural plasticity is defined as the brain's ability to generate and modify neural circuits as a result of experience. The brain is continuously creating new neuronal pathways and altering existing ones in order to adapt to new experiences, learn new information and create new memories. These changes in neural organization may account for various forms of behavior modification, which include adaptation to a mutable environment, various forms of learning and memory and compensatory adjustments in response to functional losses. These changes are a basic requirement for learning and behavioral adaptation in complex organisms (Cowansage, LeDoux, & Monfils, 2010). This dynamic remodeling depends on the ability of environmental stimulation to influence both gene expression and protein activation.

The most well know form of neuronal plasticity is synaptic plasticity. Synapses often have a remarkable capacity for short-term physiological changes (lasting milliseconds to minutes) that refer to activity-dependent modifications of the strength or efficacy of synaptic transmission (at preexisting synapses). Long-term changes (lasting days) can give rise to further physiological changes that lead to anatomical changes, including pruning of preexisting synapses and even growth of new ones (Citri & Malenka, 2008). In this form of plasticity, either the amount of neurotransmitter released from the presynaptic terminal, or its receptor in the postsynaptic neuron are modulated (Colicos & Syed, 2006). Synaptic plasticity possesses a crucial role in the capacity of the brain to incorporate transient experiences into persistent memory traces.

2.1. MOLECULAR MECHANISMS UNDERLYING NEURONAL PLASTICITY AND ITS INFLUENCE ON MEMORY FORMATION

The behavior of neural circuits depends on the pattern of synaptic weights that connect individual neurons and consequently define the circuit (Citri & Malenka, 2008). This idea was developed during years, when in the late 1940s Donald Hebb postulated that associative memories are formed in the brain by a process of synaptic modification that strengthens connections, when presynaptic activity correlates with postsynaptic firing (Hebb, 1949). The experimental support for this hypothesis of physiological changes in synaptic strength emerged in 1973 from Timothy Bliss, Tony Gardner-Medwin and Terje Lomo study's. They reported that a repetitive high-frequency stimulation of excitatory synapses caused an enhancement of synaptic transmission between the stimulated axons and the dentate areas of the hippocampus that could last for hours or even days (Bliss & Lomo, 1973). This phenomena, known as long-term potentiation (LTP) has been object of intense investigation due its important role in molecular and cellular mechanisms by which memories are formed (Martin et al., 2000; Pastalkova et al., 2006; Whitlock et al., 2006). LTP and long-term depression (LTD) are the most extensively studied physiological models of memory formation. The three well-described characteristics of LTP – cooperativity, associativity and durability – have been identified as solid arguments that support the hypothesis that LTP may be a biological substrate for, at least, some forms of memory (Nicoll, Kauer, & Malenka, 1988)

Furthermore, similar to memory, LTP can be generated rapidly and is strength prolonged by repetition. The majority of experimental work aimed to understand the mechanisms of LTP has been performed on excitatory synapses, using high-frequency bursts (tetani), specifically on the synapses between Schaffer collateral and commissural axons and the apical dendrites of CA1 pyramidal cells (Citri & Malenka, 2008; Malenka & Nicoll, 1999).

There are two major types of ionotropic glutamate receptors that contribute to postsynaptic response at glutamatergic synapses: AMPA and NMDA receptors; which are often found co-localized on individual dendritic spines. During low-frequency synaptic transmission, glutamate binds to AMPA receptor which has a channel permeable to monovalent cations (Na^+ and K^+) and provides the majority of inward current for generating synaptic responses when the cell is close to its resting potential (Figure 1). However, the triggering of LTP requires the activation of NMDA

receptors, a subtype of glutamate receptors, which are voltage-dependent and remain blocked by extracellular Mg^{2+} (Nowak et al., 1984). Activation of NMDA receptor dissociates Mg^{2+} from its binding site allowing the influx of Ca^{2+} and Na^{+} to enter the dendritic spine (Lynch et al., 1983; Malenka et al., 1988) (Figure 1b). This Ca^{2+} influx through NMDA receptors is responsible for initiating LTP by activating protein kinases – CaMKII and PKA, and producing cAMP (Citri & Malenka, 2008; Elgersma & Silva, 1999; Lynch et al., 1983). Initially, these kinase proteins phosphorylate receptors and alter the intrinsic proprieties of ligand-gated ion channels; subsequently, they activate local protein synthesis at the synapse and lead to intracellular signaling into the nucleus (via transcription factors), thereby altering gene expression (Alberini et al., 1995; Goelet et al., 1986).

Changes in synaptic strength induced by LTP can be divided into two temporally and mechanistically distinct phases:

- Early phase of LTP (E-LTP) – involves modifications of preexisting synapses, as a result of rapid Ca^{2+}influx through NMDA receptor, and subsequent protein phosphorylation events; induces an increase in synaptic efficacy that lasts for 1-2 hours (Malenka & Nicoll, 1999; Malenka & Bear, 2004; Pang & Lu, 2004).
- Late phase of LTP (L-LTP) – requires activation of cAMP-dependent protein kinase (PKA) and the transcription factor CREB (Kandel, 2001) leading to *de novo* RNA transcription, new protein synthesis and structural changes at synapses; induces an increase in synaptic efficacy lasting over hours or days (Lüscher et al., 2000).

The maintenance of LTP requires pre- and post- synaptic changes that include an increase in neurotransmitter release and a modification of AMPA receptors (Malenka & Nicoll, 1999).

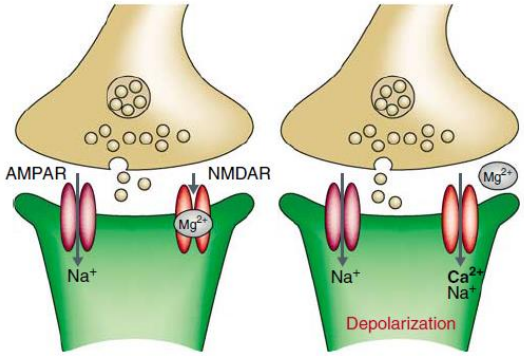

Figure 1 | Model of synaptic transmission at excitatory synapses (Citri and Malenka, 2008)

Recently, and elegant study of Nabavi and colleagues showed that fear conditioning – a type of associative memory – can be inactivated by LTD mechanisms and reactivated by LTP, supporting a causal link between synaptic plasticity and memory (Nabavi et al., 2014).

In zebrafish, few studies have been done so far in this field. In 2004, Nam and colleagues showed for the first time that NMDA receptors are synaptically activated and required for induction of LTP in zebrafish telencephalon (Nam, Kim, & Lee, 2004). In zebrafish telencephalon, a repetitive high-frequency stimulation of the connections between Dl and Dm induces a LTP that can be blocked by a NMDA receptor competitive antagonist (APV) (Ng et al., 2012). Further studies have shown the role of NMDA receptors on memory tasks performance, since the uncompetitive antagonist of NMDA receptors MK-801 prevented memory formation (Blank et al., 2009). These findings suggest that molecular and cellular mechanisms underlying learning and memory can be shared by mammals and zebrafish.

2.2. *BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)*

The brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family of trophic factors. It has multifaceted functions in CNS such as neuronal survival, differentiation, synaptic plasticity and dendritic remodeling (McAllister, Lo, & Katz, 1995; Schinder, Berninger, & Poo, 2000). Among neurotrophins, BDNF and its major receptor TrkB, have the most abundant and widespread expression in the developing and adult brain (Murer, Yan, & Raisman-Vozari, 2001). Secretion of BDNF requires previous expression of BDNF mRNA and the subsequent translation into pre-proBDNF protein.

The BDNF protein is synthesized as a precursor (pre-proBDNF protein) resulting after cleavage in a 32KDa proBDNF protein. ProBDNF is further processed until it is secreted into the extracellular space. This isoform can follow two distinct pathways to leave the intracellular compartment: either the constitutive or the regulated pathway. In the constitutive pathway, proBDNF isoform is secreted as proBDNF and cleaved extracellulary by proteases; whereas in the regulated pathway proBDNF is cleaved intracellulary by enzymes like furin or pro-convertases and originate matureBDNF (14kDa) (Cunha, Brambilla, & Thomas, 2010; Lessmann, Gottmann, & Malcangio, 2003). The biological function of BDNF is mediated by the binding of these secreted homodimeric proteins either to their cognate tropomyosin related kinase (TrkB) receptor or to the common neurotrophin receptor $p75^{NTR}$ (Lessmann et al., 2003). Once released, proBDNF binds preferentially to pan neurotrophin receptor ($p75^{NTR}$) and matureBDNF binds preferentially to TrkB receptors. BDNF is secreted both pre- or post-synpatically in an activity-dependent manner (Pang & Lu, 2004). TrkB and $p75^{NTR}$ play different roles in BDNF function, activating different intracellular messenger cascades and producing distinct cellular responses. TrkB initiates three major cascades of signaling pathways: PLCγ, PI3K and ERK/MAPK (Cunha et al., 2010), which ultimately lead to the phosphorylation and activation of CREB that mediates transcription of genes essential for survival and differentiation of neurons. $p75^{NTR}$ activation initiates two cascades of signaling pathways: JNK or NF-kB responsible for apoptosis and cell survival, respectively (Figure 2).

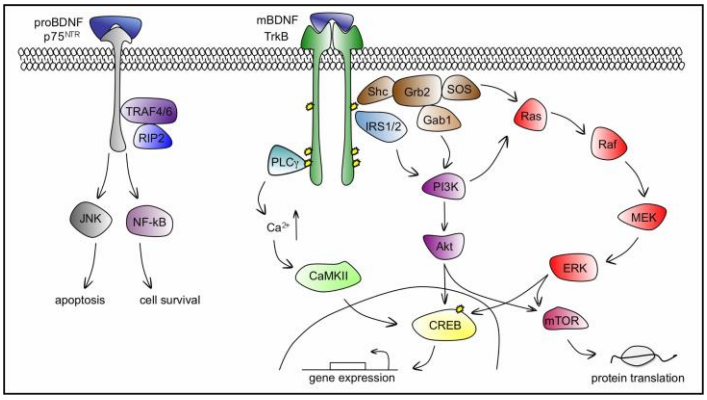

***Figure 2* | Model of BDNF-p75NTR and BDNF-TrkB intracellular signaling pathways** (Cunha et al., 2010)

In rat and mice BDNF mRNA is widely distributed throughout the CNS and its presence is correlated with local of protein synthesis. These brain areas include: hippocampus (with highest expression levels), cerebral cortex, thalamus, hypothalamus, olfactory bulb, amygdala, cerebellar granule cell layer and spinal cord (reviewed in: Edelmann, Leßmann, & Brigadski, 2013).

Several studies in teleost fish reported a structural and functional conservation of the amino acid sequence of BDNF throughout evolution. Götz and colleagues cloned BDNF mRNA from platyfish (*Xiphorus maculatus*) and found that it was 90% identical with mammalian BDNF and had identical biological activity and potency (Götz, Raulf, & Schartl, 1992). Five neurotrophin receptors have been described in zebrafish, two of which have been reported to be isoforms of the TrkB receptor, which share >90% similarity with their mammalian homologous (Martin et al., 1995). An additional BDNF function has been shown by Hashimoto and Heinrich (1997). They reported the involvement of BDNF in fin development of zebrafish, due the presence of BDNF transcripts in the pectoral fin (Hashimoto & Heinrich, 1997). In zebrafish, BDNF and its TrkB receptor are widely distributed along the brain and retina (Germana et al., 2010), playing an essential role during embryonic development (Lum, Huynh, & Heinrich, 2001).

2.2.1. *BDNF AND MEMORY PROCESSING*

It is widely believed that changes in synaptic strength of neuronal connections underlie the formation of memories. The induction of LTP – currently considered to represent the cellular model for memory – is associated with the activation of a large number of signaling cascades, including the ones activated by BDNF. Studies showing the impairment of LTP in hippocampus of heterozygous BDNF knockout animals reported, for the first time, the involvement of BDNF in LTP, which could be rescued by exogenous BDNF (Korte et al., 1995; Patterson et al., 1996). This neurotrophic factor acts through TrkB receptors either pre- or post-synaptically, to modulate LTP (Kovalchuk et al., 2002; Xu et al., 2000). Since BDNF appears to be involved in activity-dependent synaptic plasticity, several studies started to suggest its role in learning and memory mechanisms. In memory processing, BDNF acts at different molecular levels: it regulates ion channels such as Na^+ and K^+; it modulates glutamatergic receptors (NMDA and AMPA) and it affects protein synthesis by transcriptional and translational mechanisms (Bekinschtein, Cammarota, & Medina, 2013).

Accumulating evidence shows a correlation between BDNF mRNA expression and behavioral performance in memory tests (Tyler et al., 2002; Yamada & Nabeshima, 2003). Simultaneously, several studies showed that up-regulation of BDNF mRNA expression is increased in the hippocampus after memory tests such as: Morris water maze (Kesslak et al., 1998); radial maze (Mizuno et al., 2000); passive avoidance (Ma et al., 1998); and contextual fear conditioning (Hall, Thomas, & Everitt, 2000). In this context, the hippocampus appears to be involved in the regulation of memory-related BDNF activity. Recently, an increase in BDNF mRNA has been reported in perirhinal cortex 2h after exposure to novel objects (Romero-Granados et al., 2010).

3. THE SOCIAL BRAIN

In social species, where repeated interactions among the same individuals occur (i.e. social relationships), the success of these relationships depends on specific social skills. One such skill is the ability of individuals to recognize other individuals and to remember past interactions, and adjust their future behavior accordingly (Oliveira, 2013). This ability includes a wide array of cognitive processes such as attention, perception, learning, memory and decision-making. In the past years, several studies have been done to understand how these complex cognitive functions are processed in the social brain (Adolphs, 2010). It has been proposed that the mechanisms responsible for social interactions differ from those involved in non-social interactions (e.g. interactions with physical environment) (Zuberbuler & Byrne, 2006). In mammals, the neuronal circuits that evaluate social stimuli, integrate them and regulate social behavior into adaptive responses have been allocated to a network which integrates the mesolimbic reward system and to social behavior network, that together form the social decision making network (Connell & Hofmann, 2011b). According to this proposal the mesolimbic reward system is responsible for the assessment of the relative value of the social stimuli and the consequences of behaving in dissimilar forms (Connell & Hofmann, 2011a). This circuitry is characterized by massive dopaminergic projections from ventral tegmental area (VTA) to the nucleus accumbens (NAcc), but also includes reciprocal connections between hippocampus (HIP), basolateral amygdala (blAMY), vental pallidum (VP), striatum (Str), lateral septum (LS) and bed nucleus of stria terminalis/medial amygdala (BNST/meAMY) (Connell & Hofmann, 2011b). The social behavior network has been first proposed as the substrate for multiple forms of social behavior in mammals (Newman, 1999). This network compromises six brain nuclei that are reciprocally connected: the lateral septum (LS), preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VMH), periaqueductal gray/central grey (PAG/CG) and bed nucleus of stria terminalis/medial amygdala (BNST/meAMY) (Connell and Hofmann, 2011). These areas express sex-steroid hormone receptors and are implicated in a wide range of social behaviors such as parental care, aggression, mating and sexual behaviors, social recognition, affiliation, responses to social stressors and communication (Goodson et al., 2005; Newman, 1999). These multiple forms of social behavior are fundamental and evolutionarily ancient properties of most animal taxa, and as such the brain regions regulating these behaviors are expected to be highly conserved across vertebrates. The

core nodes of Newman's social behavior network were initially proposed for mammals; however, this framework has been expanded to reptiles, birds and teleosts (Connell and Hofmann, 2011; Goodson et al., 2005). Although the social behavior network and the mesolimbic reward system have been studied as separate circuits, they are anatomically linked by connections between several brain regions and share two nodes: the LS and the BNST. These two circuits complement each other by regulating both the evaluation of the valence and salience of external stimuli and the behavioral output (Connell & Hofmann, 2011b).

3.1. HIPPOCAMPUS

The hippocampal formation is one of the most studied neuronal systems in the brain. Its role in memory formation has been studied at almost every level of analysis since the discovery of the patient H.M. with hippocampal damage (Scoville & Milner, 1957). In general, patients with damaged hippocampus show an impairment of new explicit memory acquisition, whereas short-term memory, priming and procedural learning are preserved (reviewed in Bird & Burgess, 2008). The hippocampus appears to have a crucial role in short- and long-term memory (O'Keefe and Nadel, 1978; Anderson et al., 2007).

In nature, the ability to remember environmental information, recall the localization of rewarding stimuli and the identification of familiar conspecifics is clearly adaptive. Several studies have demonstrated the function of hippocampus in different vertebrate taxa (Connell & Hofmann, 2011b; Rodríguez et al., 2002). Although the vertebrates' forebrain shows an impressive range of morphological variation and specialized adaptations, the close functional similarity of this structure allows the establishment of homologies between mammals and other vertebrates (reviewed in (Connell & Hofmann, 2011a). Developmental studies demonstrate that, contrarily to what happens in mammals, in teleosts there is an eversion of the dorsal part of the neural tube (pallium), resulting in a divergent organization of the mediolateral telencephalon from that observed in mammals (Mueller, Wullimann, & Guo, 2008; Mueller & Wullimann, 2009). In teleosts, the lateral zone of the dorsal telencephalic area (Dl) is currently considered to be the homolog of the mammalian hippocampus (Portavella et al., 2002; Rodríguez et al., 2002; Wullimann & Mueller, 2004). This homology is also supported by behavioral studies that have shown that: Dl ablation leads to impairment in spatial learning acquisition and retention (López et al., 1998); spatial learning acquisition is correlated with an increment of cellular activity in Dl (Vargas et al., 2000); and these effects are similar to those of hippocampal lesions in mammals.

The role of the hippocampus in recognition memory (e.g. object recognition) has been controversial. A study in non-human primates showed a positive correlation between the percentage of damage to the hippocampus and scores on portions of the recognition performance test, suggesting that, the greater the hippocampal damage, the better the recognition (Murray & Mishkin, 1998). In contrast, a convergence of studies using this task show that hippocampal lesions produce recognition memory impairment in monkeys (Beason-Held et al., 1999; Zola et al., 2000),

humans (Reed & Squire, 1997) and rodents (Hampson et al., 1999). Recently, Hitti and Siegelbaum, reported that inactivation of CA2 pyramidal neurons caused a pronounced loss of social memory (Hitti & Siegelbaum, 2014).

4. SOCIAL RECOGNITION MEMORY

What animals know about each other, and how they construct and use knowledge of their social world involves at least an ability to recognize different social categories. Animals may categorize, and therefore recognize, individuals according to different social categories – species, group member, kin, age, sex, reproductive status and hierarchical status (Colgan, 1983). These characteristics can be detected through the assessment of cues that do not need to be individual-specific. Social recognition has been defined as "*the ability of individuals to categorize conspecifics into different classes (homo- vs heterospecific, same group vs different group, adult vs young, male vs female, kin vs nonkin, dominant vs subordinate, familiar vs unfamiliar) and to recall the learned idiosyncratic identity of a specific individual previously met*" (Gheusi et al., 1994). Thus, social memory refers to the ability of animals to change their social behaviors towards a conspecific as a consequence of a previous social encounter with it. To make this possible, a social memory needs to be stored during the initial encounter and retrieved during posterior encounters.

Social memory is a unique form of memory that is critical for reproduction, territorial defense, establishment of dominance hierarchies, pair bonding and allows to understand the structure, organization and evolution of social system (reviewed in (Ferguson, Young, & Insel, 2002; Gheusi et al., 1994). Therefore, the capacity to encode and recall this type of information is required in almost all organisms living in complex social systems. In mammals different species evolved different strategies to encode information: in humans, a specific visual association area (FFA) is responsible for face perception/recognition (Kanwisher, McDermott, & Chun, 1997); in non-human primates, the temporal cortex responds selectively to faces (Perrett, Rolls, & Caan, 1982); in most other mammals, social information is encoded via olfactory (pheromone signaling), auditory or visual signals (reviewed in Ferguson et al., 2002). In birds: long-tailed tits (*Aegithalos caudatus*) can discriminate between kin and non-kin individuals based on vocalizations (Sharp et al., 2005); in hens discrimination between familiar and unfamiliar conspecifics appears to be visual (Dawkins, 1995; Guhl & Ortman, 1953). In fish kin recognition occurs in *Salvelinus alpinus* (OlsÉn, Grahn, Lohm, & Langefors, 1998) and nestling recognition in the parental male bluegill *(Lepomis macrochirus)* (Neff & Sherman, 2003). Also invertebrates (e.g. insects), can distinguish between nestmate and non-nestmate kin based on olfactory cues (Gamboa et al., 1986). As mentioned,

different sensory systems are used in recognition, including olfactory sense, acoustic sense and vision.

The success of social recognition process depends on the integration of various crucial phases: 1) signaling of cues by the stimulus animal (e.g. unique odors, plumage patterns or vocalizations); 2) perception of these cues by other animals; 3) storage of information about familiar individuals cues; and, 4) using this information to discriminate between two individuals.

In laboratory conditions, social memory can be evaluated by changes of spontaneous exploratory behaviors directed towards conspecifics when an individual is re-exposed to a familiar or a novel conspecific. There are two commonly used behavioral tests to study social memory in laboratory animals: the habituation-dishabituation procedure and discrimination procedure. In the former, developed by Thor and Holloway (1982), a juvenile is placed in an adult's cage for a 5 min encounter. The adult exhibits intense social investigation activity towards the juvenile, and the duration of this investigation reflects the familiarity between the two animals. Thus, a repeated exposure to the same juvenile results in a decrease in investigation time (habituation), which is reversed if a novel conspecific is presented (dishabituation) (Thor & Holloway, 1982). This decrease in social investigation time is taken as evidence of social recognition memory. The discrimination procedure, first described by Mario Engelmann (1995), is based in a binary choice test between a novel and a familiar conspecific (Engelmann, Wotjak, & Landgraf, 1995). Similar to other non-social cognitive tests (object recognition), this paradigm allows the assessment, within the same test, of an animal's discrimination between two social stimuli. In this test, social recognition is assessed by comparing the difference in the time spent investigating the familiar vs. the unfamiliar conspecific.

Rodents are the most widely study model for this type of memory. Several studies in rodents have demonstrated their remarkable sensitivity to discriminate between familiar and unfamiliar individuals. However, this ability has a limitation in time: it can be a form of short-term memory with a limited duration of 30min to 2hrs (Thor & Holloway, 1982) or it can display robust long-term duration which persists for 24h to 7 days (Kogan, Frankland, & Silva, 2000; Moura, Meirelles, & Xavier, 2010).

4.1. NEUROBIOLOGICAL BASIS OF SOCIAL MEMORY

Social memory is an emerging topic of interest in memory research. The neurobiological bases of this type of memory are still poorly understood, with just a few studies addressing this question so far. However, there are several questions that must be addressed: which molecular and cellular mechanisms are involved in its formation? How long can it persist? Which brain areas are involved? What are the physiological differences comparative to others forms of memory?

In the last decade, research in this topic has focused on the role of synaptic plasticity and neuroendocrine responses. Pharmacological studies have shown the role of NMDA receptors, a modulator of synaptic plasticity, in social memory. Hlinák and Krejcí (Hlinák & Krejcí, 2002) showed that administration of NMDA antagonist (MK-801) impairs social recognition in rats when administered immediately after the initial encounter, lasting for more than 30 min. (Gao et al., 2009; van der Staay et al., 2011; Zou et al., 2008). In 2012, Jacobs and Tsien, analyzed how changes in the NMDA receptor composition may change social memory and behavior. They were especially interested in two subunits of NMDA receptor (NR2A and NR2B) that are present in excitatory neurons in the forebrain. They found that the NR2B subunit enhances learning and memory abilities (Jacobs & Tsien, 2012).

Studies conducted in the past decade have yielded several insights about neuroendocrine regulation of social recognition by the neuropeptides oxytocin (OT) and arginine-vasopressin (AVP). OT has been recognized as an important modulator of various aspects of social behavior. OT knockout mice have social memory deficits (Ferguson et al., 2000). In female rats, intra-cerebro-ventricular administration of an OT antagonist impaired social recognition (Engelmann, Ebner, Wotjak, & Landgraf, 1998). Similarly, in male rats administration of low doses of OT after an initial encounter reduced the social investigation upon a second presentation 2hrs later; and this effect could be reversed by administration of an OT receptor antagonist (Benelli et al., 1995). Supporting the involvement of OT in social recognition, an oxytocin receptor knockout mice model showed deficits in social memory, displaying equal levels of investigation of both novel and familiar conspecifics (Lee et al., 2008; Takayanagi et al., 2005). In this context, the most consistent data regarding OT and social memory come from a series of studies focused on the medial amygdala (Choleris et al., 2007; Ferguson et al., 2001). Lukas and colleagues (2013), described the functional involvement of OT in the maintenance and retrieval of social and non-social memory. During

retrieval, in the lateral septum, they found an increase in OT levels that were not present during acquisition an maintenance phases. The posterior blockade of OT activity by an OT receptor antagonist, immediately after acquisition, showed an impairment of social memory (Lukas, Toth, Veenema, & Neumann, 2013). In contrast, non-social memory (object discrimination) was not affected by OT receptor antagonist, indicating that oxytocin is mainly required for memory formation in a social context (Lukas et al., 2013). Recently, Mesic and colleagues, investigated how Gq-protein coupled metabotropic glutamate receptor (mGluR5) and OT receptor affect social memory. Using a KO for these receptors in the lateral septum, they found that the mGluR5 KO did not affect social memory, while the OT receptor KO significantly impaired preference for social novelty. In contrast, non-social memories (object recognition and fear conditioning) were not affect by these genetic manipulations (Mesic et al., 2015). Like OT, acute manipulation of the AVP system also revealed its importance for social recognition memory. In more detail, peripheral administration of AVP enhances recognition responses (Le Moal et al., 1987). Furthermore, the ventricular administration of a selective AVP (V1a) receptor antagonist inhibits recognition (Le Moal et al., 1987). In V1aR KO mice social memory is completely impaired; whereas in V1bR KO mice it is only partially impaired (Wersinger et al., 2002).

Performance on social recognition memory requires the ability to identify and remember information about individuals. In rodents this information can be stored for up to 60 minutes (short-term memory), or maintained for longer periods of time (24h: (Richter, Wolf, & Engelmann, 2005); 7 days: (Kogan et al., 2000)), reflecting long-term memory. A considerable amount of evidence shows that long-term memory (but not short-term memory) depends on *de novo* protein synthesis. Kogan and colleagues, showed that lesions in the hippocampus disrupt social recognition, and long-term memory was dependent on protein synthesis and cyclic AMP responsive element binding protein function (CREB) (Kogan et al., 2000). In this case, CREB seems to be a gain control device that regulates the expression of genes necessary for memory consolidation (Silva et al., 1998). However, this type of memory requires two stages of protein synthesis: the first stage takes place 1-2h after sampling and is paralleled by an increase in synthesis of *c-fos* in various brain structures; the second stage takes place between 6-7h after sampling and can be linked with synthesis of proteins that are necessary for enhanced intercellular communication (Richter et al., 2005). However, animals treated with anisomycin (protein synthesis inhibitor) between 9-15 hrs after sampling, showed a block of long-term memory (Wanisch,

Wotjak & Engelmann, 2008). Therefore, these studies show that social memory shared characteristics with other hippocampus-dependent memories.

In rodents, social recognition memory is based mainly on olfactory cues present in the anogenital area (reviewed in: Carr et al., 1976; Ferguson et al., 2002; Gheusi et al., 1994; Popik et al., 1991). It has been demonstrated that sensory inputs from olfactory cues (e.g. urine samples) are sufficiently to promote social recognition.

4.2. ZEBRAFISH AS A MODEL FOR STUDY SOCIAL MEMORY

In this thesis, zebrafish (*Danio rerio*) were used as an animal model. Zebrafish is a small tropical freshwater teleost distributed throughout South and Southeast Asia. In 1930, zebrafish was being used as a classical developmental and embryological model (reviewed in Laale, 1977). In the last decades, *Danio rerio* have become an important model organism in developmental biology, genetics, neurobiology and biomedicine. Its qualities, such as easy breeding; easy to mimic natural conditions in captivity; short inter-generation and development time; diurnal habits; small size (2-4m standard length) and transparent larvae and eggs, made this teleost fish an excellent model for manipulation studies in laboratory (reviewed in Spence et al., 2008). Additionally, zebrafish display many genetic, neural and endocrine similarities to other vertebrates. Adult zebrafish display a wide repertoire of behaviors and exhibit some similarities in brain function with other vertebrates which lead for their use in translational studies of attention (Braida, Ponzoni, Martucci, & Sala, 2014); memory (Blank et al., 2009; Cognato et al., 2012; Williams, White, & Messer, 2002); learning (Colwill et al., 2005; Xu et al., 2007; Zala & Määttänen, 2013); anxiety (Blaser & Rosemberg, 2012); addiction (Gerlai et al., 2000; Kily et al., 2008) and stress (Barcellos et al., 2007). In the field of memory, several studies clearly demonstrate the mnemonic ability of zebrafish: olfactory conditioning (Braubach et al., 2009); shuttle box learning (Pather & Gerlai, 2009); appetitive choice discrimination (Bilotta et al.,2005); Y-maze memory task (Cognato et al., 2012); aversive reinforcement learning (Aoki et al., 2013).

Zebrafish are highly social, showing preference for the presence of conspecifics. They form aggregates called shoals that offer ecological benefits such as reduction of predation risk, and enhancement of foraging and reproductive success (Krause et al., 2000). Interestingly, interactions early in life shape juvenile shoaling choices. Thus, zebrafish do not associate randomly, rather they show visually mediated preferences for fish of a similar phenotype to the one of their rearing companions (Engeszer, Ryan, & Parichy, 2004a), demonstrating a discrimination process based on recognition. On the other hand, the evolution of social behavior requires mechanisms to avoid investing in conspecifics that are not increasing an individual's fitness, suggesting the capability of recognizing kin. Thus, zebrafish are able to recognize individuals that share the same genetic relatedness. Gerlach and colleagues (2006) proposed that this process is based on phenotyping matching: zebrafish show preference for unfamiliar kin rather than unfamiliar nonkin and prefer

familiar kin to unfamiliar kin (Gerlach & Lysiak, 2006). This ability is based on a learned olfactory and visual imprinting process that triggers 6 days post-fertilization (Gerlach et al., 2008; Hinz et al., 2013). Research on visual cues suggested that zebrafish prefer to shoal with conspecifics with same size and pattern and that females can distinguish males based on visual cues alone (Hutter, Zala, & Penn, 2011; McCann, Koehn, & Kline, 1971; Rosenthal & Ryan, 2005). These findings suggest that this behaviors are visually based. However, the use of olfactory cues is also used in conspecifics discrimination. As mentioned above, kin recognition is olfactory based (Gerlach & Lysiak, 2006). Thus, zebrafish have the ability to use both visual and chemical cues to base their social preferences. However, it remains unclear under which conditions they use each of these cues to remember specific conspecifics.

All these properties support the use of zebrafish (*Danio rerio*) as an ideal model for studying social memory mechanisms.

CHAPTER 2

Objectives

In the animal kingdom, species differ strongly in their sociality. Social species show group cohesion and social affiliation between their members, whereas asocial species show the opposite. Individuals of gregarious species interact with several others and therefore, the capacity to encode and recall social information is clearly important. Social memory (i.e. discrimination of conspecifics) is a form of memory that is crucial to perform social behaviors. Several studies have reported the occurrence of social memory in different species. These studies suggest that the amygdala is the main brain area where this type of memory is allocated; and demonstrate the essential role of oxytocin and vasopressin for this type of memory. A combination of different sensory modalities can be used in social recognition, including chemical, acoustic and visual. However, relative few studies have examined the contribution of specific sensory modalities to social recognition.

Taking all these points into account, the main goals of this study are: i) to validate the use of immediate early genes as markers of neural activity in the zebrafish telencephalon; ii) evaluate if zebrafish has the ability to recognize conspecifics, and to retain this information and recall it after 24 hours; iii) to evaluate which are the sensory cues that zebrafish uses to discriminate between conspecifics; and iv) to investigate the role of BDNF in social memory, especially in the teleost homologue of the hippocampus. To address these goals two experiments were conducted: experiment 1 addressed aim 1 and the other 3 goals were addressed by experiment 2.

CHAPTER 3

Methods

Animals housing

Adult [i.e. 10-12 months old; standard length = 3.675±0.15cm (mean±SEM)] zebrafish females (N=148) from the AB wild-type strain were used throughout this study. The animals were breed and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) and belong to a F2 generation derived from founders imported from ZIRC. Animals were kept in a water recirculation system (ZebraTec, 93 Tecniplast), at 28ºC in a 14h/light: 10h/dark cycle. The water quality was monitored every day, according to the manufacturer's instructions: nitrites <0.2ppm, nitrates <50ppm, ammonia 0.01-0.1ppm, pH = 7 and conductivity at 700 µSm. Animals were fed twice a day with GEMMA 300 and arthemia (*Artemia salina).*

Experimental procedure

This experiment was done during the dark cycle (i.e. 10pm – 7am). Before starting the experiments, during the light phase, fish were placed in individual tanks (18x15cm). During the dark phase, females were carefully caught and anaesthetized by immersion in Tricaine 1x (MS-222, Pharmaq; 500–1000 mg/L). Immediately after, females were given intraperitoneal injections of kainic acid (0.5mg/Kg, Sigma-Aldrich, USA) or saline solution (control). The injection volume (either drug or saline) was always 15µL. After the injections, animals were returned to their individual tanks.

Animals from each treatment (i.e. KA and control) were deeply anaesthetized and sacrificed at various sampling points after injection (Figure 3). The baseline group was collected and sacrificed immediately without being injected; the 0 hours group was collected, anesthetized, injected (with either kainic acid or saline) and sacrificed immediately; the 0.5 hours group was collected, anesthetized, injected (with kainic acid or saline) and sacrificed after a delay of 30 minutes. This design was maintained to all time points (0hr; 0.5hr; 1 hr; 2hr; 4hr; 8hr; 24hr and 48hr). Assays were conducted with eight fish per group.

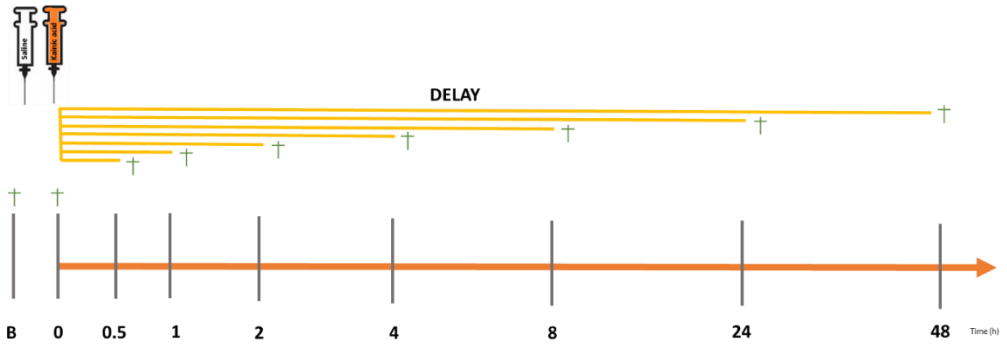

Figure 3| Schematic representation of experimental procedure. After administration of KA or saline solution, animals were sacrificed at different timepoints.

Sampling

Fish were sacrificed at each sampling point with an overdose of tricaine solution 25x (MS222, Pharmaq; 500-1000mg/L). The brain was macrodissected under a stereoscope (Zeiss; Germany) into five areas: Telencephalon (TL); Optic tectum (OT); Diencephalon (DE); Cerebellum (CB) and Brain stem (BS). The response to brain stimulation was characterized at two levels of genomic modifications: gene expression and protein levels. For this purposes, each brain was divided sagitally into two halves: one side for qPCR analysis and the other side for Western-blots (Figure 4). This procedure was balanced to control for brain lateralization.

Immediately after dissection, each brain area was collected into 50µL of QIAzol® or 100µL of HEPES buffer (10mM HEPES; 300mM KCl; 3mM MgCl2; 100mM CaCl2; 0.45% Triton X-100; 0.05% Tween-20; Protease inhibitor cocktail (ROCHE); pH 7.6) – depending on purpose – and stored at -80°C until further analysis.

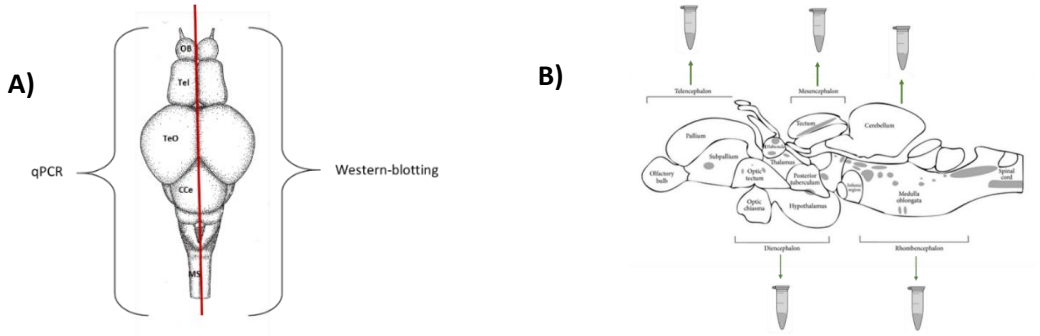

Figure 4|Brain areas collected. A) Mid sagittal cut; right side for qPCR and left side for western-blotting; B) In each half part of the brain, five macroareas were collected.

Western-blotting

Tissues were mechanically homogenized and centrifuged, in 50µL of HEPES buffer, at 13000*g* for 10 minutes at 4°C. The supernatant were collected and used for total protein quantification (Bradford assay). Samples (30 µg of total protein) were used to run the Western-blots. After denaturation (5 minutes at 95°C), protein samples were separated by SDS-PAGE, in 15% polyacrylamide gel and transferred to PVDF membranes (Bio-Rad, USA) in a semi-dry system (Trans-Blot®Turbo™System, Bio-Rad, USA), during 30 minutes at 25V and 1.0A. Membranes were cut at level of 45kDa according to protein markers. The two membranes were incubated for 1 hour at room temperature in blocking solution (5% (v/v) non-fat milk powder (Amiresco, USA) prepared in TBS-T (0.1% Tween 20). The upper part of the membrane (>45kDa) was incubated overnight at 4°C with anti-α-tubulin antibody (Sigma-Aldrich, diluted 1:1000). The lower part of the membrane (<44kDa) was incubated with anti-BDNF antibody (N-20, Santa Cruz Biotechnology, diluted 1:100). In this study, the two BDNF isoforms (mature and pro-BDNF) were evaluated. The anti-BDNF antibody recognizes both isoforms of BDNF (mature – 14kDa; precursor – 32kDa). After hybridization with the specific antibody, membranes were washed and incubated with secondary antibodies for 1 hour at room temperature. For α-Tubulin the secondary goat anti-mouse HRP was used (Sigma-Aldrich, diluted 1:1000), and the secondary goat anti-rabbit HRP was used (Santa Cruz Biotechnology, diluted 1:5000) for BDNF. Finally, membranes were washed and immunoreactive bands were detected using a chemioluminescense system (WesterBright ECL HRP substrate, GRISP). Optical density was determined using Image-J software. Anti-α-tubulin was used as loading control and the results were expressed after normalization.

Stripping and re-probing membranes

In order to quantify C-FOS proteins, membranes were re-probed to an additional primary antibody (anti-c-fos-antibody, Santa Cruz Biotechnology). ECL was removed by washing the upper part of the membranes (>44KDa) with TBS-T for 30 minutes. After this step, anti-α-tubulin primary antibody was removed by washing the membrane with a stripping buffer (Tris-HCl pH 6.8; ultra-pure water; β-mercaptoethanol; SDS 10%), during 45 minutes at 50°C. Membranes were washed again, under running water tap for 2 hours. Membranes were blocked with 5% non-fat milk in TBS-T, 1 hour, and incubated with anti-cfos-antibody (1:50), overnight at 4°C. Secondary antibody was incubated with anti-rabbit for 1 hour at room temperature.

RNA isolation, cDNA synthesis and real-time quantitative PCR amplification

For the RNA isolation, the total RNAs were extracted from each brain macroarea using an RNeasy Lipid Tissue Mini Kit (Quiagen, USA). Tissues were disrupted and homogenized mechanically in 50µL of QIAzol® and incubated during 7 minutes before the addition of 100µL of chloroform. Samples were incubated for 5 minutes at room temperature and then centrifuged at 13000*g* for 30 minutes at 4°C. After separation of the aqueous phase, the RNA was precipitated in 50µL of ethanol (70% solution) and the pellets were successively washed with buffers (ethanol and water). At the end, samples were centrifuged and resulting pellets were resuspended in 30µL of RNase-free water (Lonza, Switzerland). RNA quality and integrity was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, UK). RNA samples were stored at -80°C until further use.

The first-strand cDNA was synthesized using an iScript™ cDNA synthesis kit (Bio-rad, USA), that is based on RNase H^+, oligo (dt) and random hexamer primers in the reaction mix. 15µL of isolated RNA were mixed with 4µL of 5xiScript reaction mix and 1µL of iScript reverse transcriptase. The reaction was performed at 25°C for 5 minutes (primer annealing), followed by 60 minutes at 42°C (cDNA synthesis) and finally 5 minutes at 85°C (denaturation of reverse transcriptase). Samples were cooled to 4°C and then stored at -20°C.

The relative expression of target (*c-fos* and *bdnf*) and housekeeping (elf-1a) genes was assessed using real-time qPCR. For this analysis, 2µL of cDNA were added to 4µL of SYBR green PCR master mix (Applied Biosystems, Life Technologies, USA), 0.15µL of each primer (Table1) and 1.7µL of RNase-free water (Lonza, Switzerland). For gene expression quantification, the reaction started with a denaturation phase (5 minutes at 95°C); followed by an amplification and quantification phases (40 cycles 30 seconds at 95°C, 30 seconds at primer specific annealing temperature (Table 1) and 30 seconds at 72°C); and a melting curve assessment phase (30 seconds at 95°C; 30 seconds at 55°C followed by an 55-95°C with a heating rate of 0.5°C/s). All reactions were run in triplicate.

qPCR was performed in ABI7900HT (Applied Biosystems, Life Technologies, USA). Afterwards, the collected data was analyzed using Sequence Detection Systems (SDS 2.4) (Applied Biosystems, Life Technologies, USA).

Table 1**|Primer sequences, annealing temperature and efficiency**. All primers were commercially synthesized (Sigma-Aldrich, Germany).

PRIMER	PRIMER FORWARD SEQUENCE	PRIMER REVERSE SEQUENCE	ANNEALING TEMPERATURE (°C)	EFFICIENCY (%)
elf-1a	5´CAAGGAAGTCAGCGCATACA3´	5´TCTTCCATCCCTTGAACCAG3´	59	96,2%
c-fos	5´CCGATACACTGCAAGCTGAA3´	5´CGGCGAGGATGAACTCTAAC3	59	99,0%
bdnf	5´GCTGCCGAGGAATAGACAAG3´	5´CTGCCCCTCTTAATGGTCAA3´	61	99,2%

qPCR data processing

The threshold cycle (Ct) represents the detectable fluorescence signal above background and results from the accumulation of amplified product. Ct was measured in the exponential phase and therefore was not affected by possible limiting components in the reaction. A Ct value was defined for each gene, along the exponential phase, with the subtraction of higher value before the linear phase to the minimum value of the exponential phase. Afterwards, gene expression levels were assessed using: 2^(Ct housekeeping – Ct target gene). It was assumed that the efficiency of the genes were 100%.

Statistics analysis

Normality was verified using the Shapiro Wilk's W test and taking in consideration skweness and kurtosis values. Homoscedasticity was tested using Levene's test. Comparisons between control (saline solution) and treatment (kainic acid) group at each sampling point were performed using an ANOVA. To evaluate the changes in gene expression or protein levels in response to the treatments, two-way ANOVAs followed by planned comparisons were performed. All data are represented as mean ±SEM. Statistical significance was set at $p<0.05$.Outlier's analysis was performed using Grubbs test. All statistical analysis were performed using SPSS Statistics 21® (IBM, USA).

Animals housing

Adult [i.e. 7-10 months old; weight = 0.35 ± 0.05g (mean ± SEM); total length = 3.7 ± 0.17cm (mean ± SEM)] zebrafish males (N=48) from the Tubingen (Tu) strain were used as experimental subjects. Fish were breed and held at Instituto Gulbenkian de Ciência (IGC, Oeiras, Portugal) and belong to a F2 generation descendent, from founders imported from ZIRC. Fish were housed in groups of eight individuals per tank (40x20x30cm) at 28ºC and a 14h light/ 10h dark cycle. The water quality was monitored every week keeping nitrites (<0.2ppm), nitrates (<50ppm) and ammonia (0.01-0.1ppm) in optimal values. The pH and conductivity were maintained at 7 and 700 µSm, respectively. Fish were feed twice a day with GEMMA 300 and arthemia (*Artemia salina).*

Adult males of the same strain, but belonging to different crosses (i.e. with different genetic background) were used as social stimuli. Focal and stimuli fish were reared and housed separately, in tanks with a 8:2 sex ratio (M:F), to avoid possible familiarity effect. The stimulus fish were tested only in two separate tests during the day.

Animal tagging

In order to identify the animals individually, fish were tagged before the experiments. Fish were anaesthetized in Tricaine 1x (MS-222, Pharmaq; 500–1000 mg/L), weighed, measured for standard length and tagged. The tagging was done by inserting a 27G needle (Premier Healthcare & Hygiene, Lda) with 0.4mm in the musculature zone below the dorsal fin. The needle, containing a monofilament (Shimano, Japan) with 200µm diameter, was removed leaving the monofilament crossing the animal's body. A knot was then done at each end and painted with nail polish of different colors.

Animals were allowed to recover from the tagging procedure in individual tanks for 30 minutes, after which they were transferred to stock tanks (6L, 40x20x30cm) shared with conspecifics. The recover from surgery was assured during 7 days before the experiment started.

Experimental tests

Different tests were used to evaluate social memory and determine whether a zebrafish can distinguish between a familiar and a novel conspecific. The present study includes two types of procedures to assess social recognition: a social discrimination paradigm and a habituation-dishabituation paradigm. Both paradigms exploit the natural preference of zebrafish for novelty and were adapted from mouse studies (Engelmann et al., 1995; Thor and Holloway, 1982). An object recognition test was also performed to compare social and object recognition abilities in fish.

Each animal was exposed to the following tests: 1) object recognition (O); 2) visual social discrimination (V); 3) olfactory social discrimination (C); 4) multimodal (i.e. visual and olfactory) social discrimination (V+C); 5) habituation-dishabituation test with novel + novel individuals (N+N); 6) habituation-dishabituation test with novel + familiar individuals (N+F). Each animal did each test once, with an inter-test interval of 4 days. Between tests, fish were kept in home tanks in groups of 10 individuals that were maintained during the study.

All behavioral tests were run in a 60x20x20cm glass tank, divided into three same-size chambers (Figure 5). Each chamber was divided by a removable transparent partition which allowed stimuli visualization. The walls were surrounded with a white plastic sheet, so that no external stimuli could be seen during the experiment.

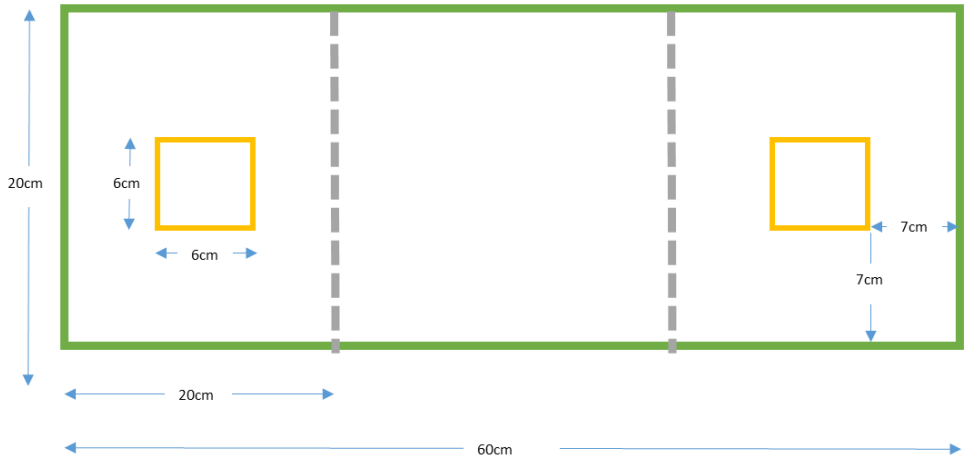

Figure 5 |Top view of experimental set-up. The grey dotted lines represent removable partitions and the yellow squares represent the localization of demonstrators' fish.

An overhead video-camera was used to monitor the focal fish behavior during the test. All behavioral tests occurred during the light phase (between 9:00 a.m. and 6:00 p.m.). The following behavioral tasks comprised a habituation, acquisition and a test phase separated by a 24 hours delay (Figure 6). Each test was divided into three parts:

Apparatus habituation

Before starting the experiment, fish were isolated in individual tanks (15x18cm) in order to control for effects of previous social experience. On day 1 of the experiment each fish was placed in the central compartment of the experimental tank and allowed to become familiar with the empty tank for 5 minutes. At the end, the animals were captured and returned to individual tank. This task was done only on the first set of tests.

Acquisition phase

In the acquisition phase (day 2), each animal was placed in the tank center and were allowed to explore spontaneously the chamber during 5 minutes. Then, the partitions were removed and the subject was allowed to move freely along the chambers, exploring objects or conspecifics, during 20 minutes. After this period, the animals were caught and returned to individual tank.

Test phase

During the test phase (day 3), each animal was placed in the tank center and was allowed to spontaneously explore the chamber during 5 minutes. Then, the partitions were removed and the subject was allowed to move freely along the chambers, discriminating between a new/familiar object/conspecific and a novel ones during 20 minutes. After this period, the animals were caught and returned to the home tank.

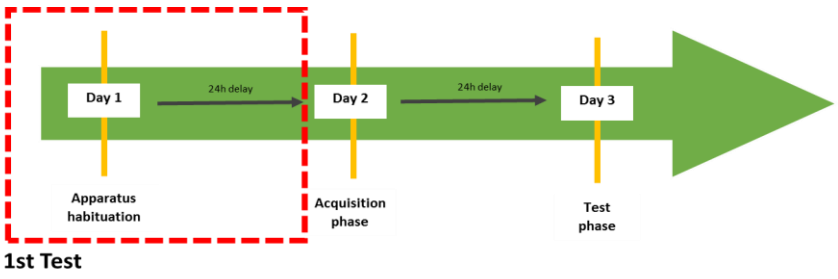

Figure 6 | Schematic representation of behavioral procedure

a) Object recognition

The object recognition test (Figure 7A) was used to evaluate the ability to distinguish between a familiar and a novel object. Plastic objects varying in shape but with the same color (red) and volume were used as stimuli. Red was selected given the natural preference of *Danio rerio* for this color (Avdesh et al., 2010). Three object shapes were used: sphere, triangular prism and cube; a behavioral test showed that zebrafish spent the same proportion of time exploring these shapes, showing no preference between them (Cognato et al., 2012). The type of stimulus (sphere, triangular prism or cube) and its positions were counterbalanced across subjects.

During the acquisition phase, two objects were placed in top end compartments of the apparatus; one on the left side and another on the right side. In the test phase, two objects were placed again in the opposite ends of the apparatus; a familiar object which has been used at day 1 and a novel object which the fish has not seen before. The two objects were placed in different parts of the tank (e.g. if at day 1 it has been in the left side, at day 2 it need to be placed in the right side).

b) Social recognition

The social discrimination paradigm is a binary choice test. Briefly, during the first 5 minutes, the experimental animal was allowed to spontaneously explore the center chamber (Figure 7B). After habituation to the center chamber, the experimental fish was allowed to freely explore two other chambers, containing two non-familiar fish in each one. On second day, experimental fish were allowed to choose between an already familiar fish and a newly introduced fish.

To understand and determine the value of sensory stimuli, the social discrimination paradigm was done combining different sensory stimuli. The study combined 3 tests for sensory assessment: visual value, olfactory value and a combination of visual and olfactory stimuli. The only difference between these tests was the type of sensory interaction between the focal and the stimulus fish.

The stimulus fish (familiar and non-familiar) were placed inside different types of transparent acrylic parallelepipeds (6x6x20cm) that allowed water exchange, auditory, visual and

olfactory interaction; while prevent direct contact between experimental and stimulus fish. In the **visual test**, the parallelepiped did not possesses orifices in order to prevent chemical communication; in the **olfactory test**, the parallelepiped was covered with white sheet paper, to prevent visual communication, but contained several orifices on the walls that allow the passage of olfactory cues; in the **visual and olfactory** test, the transparent parallelepiped contained several orifices on the walls. The use of parallelepipeds prevented direct interactions between experimental and the stimulus fish. Prior to the experiment, all stimulus fish were gently habituated to being in parallelepipeds and fish showed no obvious signs of distress from being in them.

The habituation/dishabituation procedure is widely used in social recognition in rodents (Choleris et al., 2009). Briefly, on day 1 a stimulus fish was placed into the experimental apparatus (Figure 7B), inside a transparent acrylic parallelepiped, in one of the three chambers. The experimental fish was placed on the center chamber, exploring it for 5 minutes. Subsequently, fish were left to freely explore the three chambers for 20 minutes. On second day, either the same or a novel stimulus fish was placed back into transparent parallelepiped under investigation of experimental fish for 20 minutes. In this paradigm, social recognition is inferred from change in the behavior of the experimental fish upon stimulus fish. In rodent studies, upon repeated exposures, animals shows a reduction in their social investigation (habituation) compared with animals that were presented with novels conspecifics (dishabituation) (Choleris et al., 2009).

c) Sociability

The sociability test focuses on the behavior of one animal towards another. It includes the study of the animal's interest in a social stimulus (a conspecific placed in one of the outer chambers), versus a neutral stimulus (an empty chamber). The interest is measured by assessing the time spent in the same chamber or in close proximity to the stimulus fish.

In this study, the sociability test was adapted from the habituation-dishabituation paradigm. In the first day of this test, the focal fish was placed in the middle chamber and allowed to explore for 20 minutes one stimulus fish. The stimulus fish was enclosed in a small parallelepiped and stablished visual and olfactory contact with the focal fish, as described before

(Figure 7-C). Results obtained from the first day of habituation-dishabituation paradigm were used as an index for sociability.

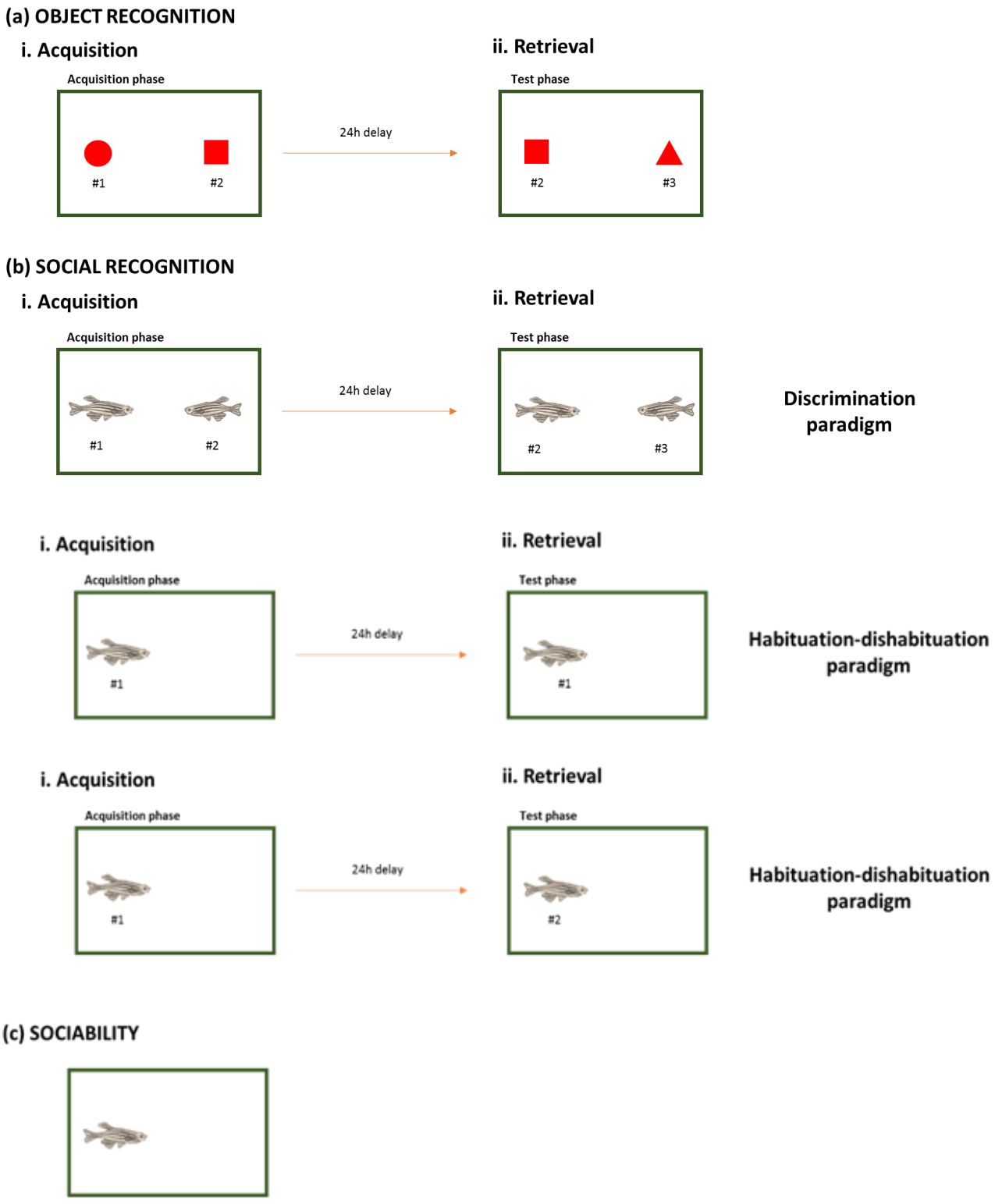

Figure 7 | Diagrams of the recognition memory tasks. a) Object discrimination test; b) Social discrimination test and c) sociability test.

Fish behaviors were subsequently analyzed using the video-tracking system *Ethovision®* XT8 (Noldus, Netherlands). As a measure of stimuli exploration, the proportion of time spent in each chamber was compute using the following formula:

$$\text{Exploration time} = \left(\frac{(tnovel + tfamiliar)}{(tnovel + tfamiliar + 2(tneutral))} \right)$$

As a measure of stimuli preference, the proportion of time spent in the target stimulus chamber was compute using the following formula:

$$\text{Stimulus preference} = \left(\frac{(tfamiliar)}{(tnovel + tfamiliar)} \right)$$

To evaluate differences in the habituation-dishabituation paradigm, we calculated a "recognition ratio", which evaluated the value of investigation duration for the test trial (day 2) divided by the sum of the initial (day 1) and the test (day 2) trial investigation duration. A recognition ratio of 0.5 indicates that there was no difference between the initial and the test investigation and therefore was an absence of recognition.

$$Recognition\ ratio = \frac{day\ 2}{day\ 2 + day\ 1}$$

Brain tissue sampling

Two hours after the end of behavioral experiments, animals were sacrificed with an overdose of Tricaine 25X (MS-222, Pharmaq; 300-400ppm). The heads were immediately removed and incorporated in Optical Cutting Temperature (O.C.T.) (Tissue-Tek®, Sakura, Netherlands), at -80°C. Whole-heads were then sliced coronally in 150µm sections using a cryostat (Leica, Germany) and mounted on glass slides (Thermo Scientific, USA). Regions of interest in the brain were microdissected under a stereoscope (Zeiss; Germany) using a modified 27G needle (Terumo, Japan) with an internal diameter of 200µm. The zebrafish brain atlas (Wullimann et al., 1996) was used to identify the regions of interest: medial zone of dorsal telencephalic area (Dm), putative homologue of the tetrapod amygdala and lateral zone of dorsal telencephalic area (Dl) putative homologue of the tetrapod hippocampus. The dissected tissue was collected into a lysis buffer (QIAzol, Quiagen, USA) and stored at -80°C until further analysis.

RNA isolation, cDNA synthesis and real-time quantitative PCR amplification

The total RNA was extracted from each brain region using an RNeasy Lipid Tissue Mini Kit (Quiagen, USA). Tissues were disrupt and homogenize mechanically in 50µL of QIAzol® and incubated during 7 minutes before the addition of 100µL of chloroform. Samples were incubated for 5 minutes at room temperature and then centrifuged at 13000*g* for 30 minutes at 4°C. After separation of the aqueous phase, the RNA was precipitated in 50µL of ethanol (70% solution) and the pellets were successively washed with buffers (ethanol and water). At the end, samples were centrifuged and the resulting pellets were re-suspended in 30µL of RNase-free water (Lonza, Switzerland). RNA quality and integrity was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, UK). RNA samples were stored at -80°C until further use.

The first-strand cDNA was synthesized using an iScript™ cDNA synthesis kit (Bio-rad, USA), that is based on RNase H^+, oligo (dt) and random hexamer primers in the reaction mix. 15µL of isolated RNA were mixed with 4µL of 5xiScript reaction mix and 1µL of iScript reverse transcriptase. The reaction was performed at 25°C for 5 minutes (primer annealing), followed by 60 minutes at 42°C (cDNA synthesis) and finally 5 minutes at 85°C (denaturation of reverse transcriptase). Samples were cooled to 4°C and then storaged at -20°C.

The relative gene expression of target genes (*bdnf*) and housekeeping (*elf-1α*) were assessed using real-time qPCR. For this analysis, 2µL of cDNA were added to 4µL of SYBR green PCR master mix (Applied Biosystems, Life Technologies, USA), 0.15µL of each primer (Table1) and 1.7µL of RNase-free water (Lonza, Switzerland). For gene expression quantification, the reaction started with a denaturation phase (5 minutes at 95°C); following an amplification and quantification phase (40 cycles 30 seconds at 95°C, 30 seconds at primer specific annealing temperature (Table 1) and 30 seconds at 72°C); and a melting curve assessment (30 seconds at 95°C; 30 seconds at 55°C followed by an 55-95°C with a heating rate of 0.5°C/s). All reactions were run in triplicate.

PCRs were performed in ABI7900HT (Applied Biosystems, Life Technologies, USA) and the collected data were analyzed using Sequence Detection Systems (SDS 2.4) (Applied Biosystems, Life Technologies, USA).

Statistics analysis

Normality was verified to all data performing Shapiro Wilk's W test and evaluated skweness and kurtosis values. Homoscedasticity was verified through Levene's test.

Comparisons between the preference scores obtained for day 1 (i.e. novel fish 1 vs. novel fish 2) or day 2 (e.g. novel vs. familiar fish) were performed using a two-tailed Student's t test. The exploration scores obtained for day 1 were compared to day 2 using a two-tailed Student's test. Correlations between the first day of habituation-dishabituation paradigm (N+N) and the second day of V+C memory discrimination test scores were performed using a Pearson correlation. Comparisons of gene expression between different behavioral tasks (e.g. visual vs. olfactory in Dm) were assessed using a one-way ANOVA, followed by Tukey's *post hoc* test. No comparisons were made between areas, since the amount of brain tissue for each area was not similar. All data are represented as mean ±SEM. Statistical significance was set at $p<0.05$. Outliers' analysis was performed using Grubbs test. All statistical analysis were performed using SPSS Statistics 21® (IBM, USA).

CHAPTER 4

Results

To study the temporal response of IEG's (*c-fos* and *bdnf*) to kainic acid injection, an analysis of gene expression and protein levels was performed. The following results show the gene expression and protein levels of *c-fos* and *bdnf* in the telencephalon of zebrafish along the sequential sampling points.

1.1. Effects of Kainic acid on c-fos and bdnf gene expression in the telencephalon

There was a main effect of sampling time on *c-fos* levels ($F_{(8,127)}$=31.485; p=0.000). An up-regulation of *c-fos* gene expression was observed 30 min. after the administration of either KA or saline solution (Figure 8-A; Table 2).

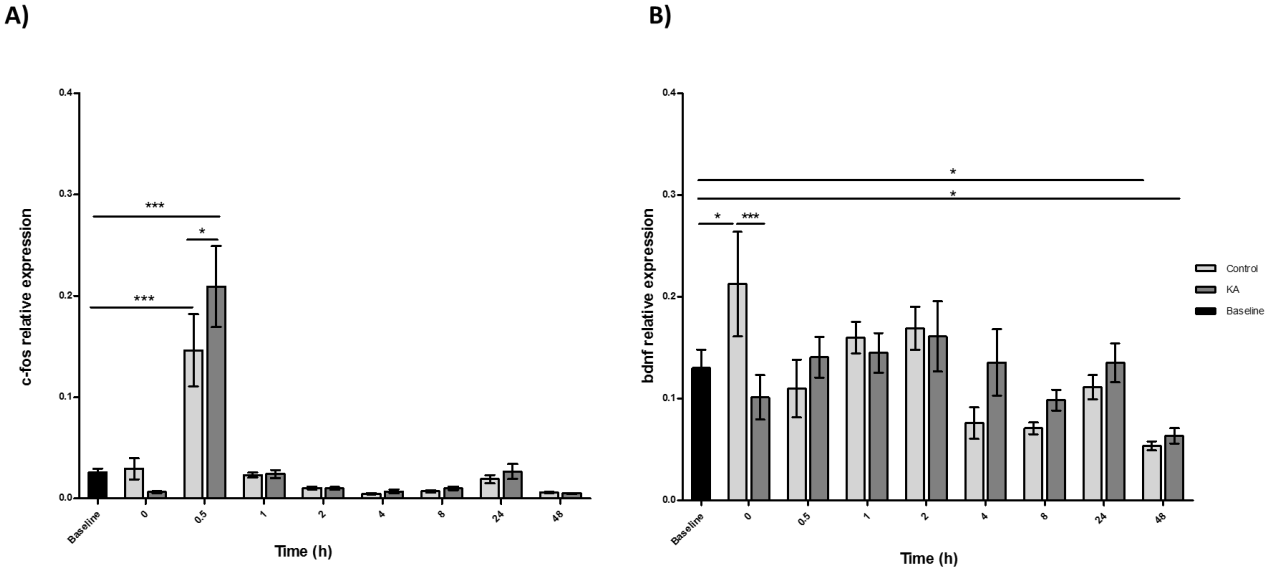

Figure 8 |Temporal changes in IEG's expression after a treatment with kainic acid. Animals were injected with either saline or kainic acid solution and sacrificed immediately, 30 min., 1hr, 2hr, 4hr, 8hr, 24hr or 48hr after injections. The time course, after either saline or KA administration, for increases in relative levels of mRNA in telencephalon are shown for *c-fos* (A) and *bdnf* (B). Acute intraperitoneal kainic acid (0.5mg/Kg) increases (A) *c-fos* expression 30 minutes after treatment; (B) *bdnf* expression 0 minutes after saline solution administration. The results are the mean±SEM of seventeen different experiments performed in independent animals. *p≤0.05; ***p≤0.001.

Nevertheless, along the timeline no significant differences were found between the kainic and the control group (Table 2). The up-regulation of *c-fos* at 30 minutes was followed by an abrupt decrease in gene expression values that were maintained near baseline levels for 48 hours.

Regarding *bdnf*, there was a main effect of sampling time in *bdnf* levels ($F_{(8,127)}$=4.244; *p*=0.000) (Figure 8B). The saline solution only induces an up-regulation of *bdnf* gene expression at 0h after administration (Table 2). However, 48h after administration of the saline solution, a decrease in expression levels of *bdnf* gene was found. KA induced a down-regulation of *bdnf* 48h after administration, with no significant differences relative to the baseline in the remaining sampling points (Table 2). The up-regulation of gene expression induced by saline solution at 0hrs was not observed when KA was administered at the same sampling point, and there was a significant difference between these two groups at this sampling point ($F_{(1, 112)}$ = 11.49; p=0.001). No other significant differences between the control and treatment groups were found in the subsequent sampling points (Table 2).

Table 2| ANOVA analyses for *c-fos* and *bdnf* gene expression after a treatment with kainic acid (Ka) and saline solution (Ct) at different sampling timepoints (0h, 0.5h, 1h, 2h, 4h, 8h, 24h and 48h). Main effects and results from planned comparisons are present in this table. Abbreviations: B, baseline; Ct, control group; Ka, Kainic acid group. *p<0.005.

		c-fos		***bdnf***	
		F	***p-value***	**F**	***p-value***
	Sampling time	31.485	***0.000**	4.244	***0.000**
	Treatment	0.226	0.798	0.35	0.966
	Sampling time x Treatment	17.061	***0.000**	3.365	***0.000**

Planned comparisons I (Baseline x Sampling time x Treatment)

	Control		**KA**		**Control**		**KA**	
	t-value	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**
B x 0h	0.182	0.856	-1.007	0.316	2.544	***0.012**	-0.884	0.378
B x 0.5h	6.289	***0.000**	9.570	***0.000**	-0.593	0.554	0.329	0.743
B x 1h	-0.145	0.885	-0.083	0.934	0.955	0.341	0.462	0.645
B x 2h	-0.802	0.424	-0.829	0.409	1.207	0.230	0.960	0.339
B x 4h	-1.070	0.287	-0.947	0.346	-1.604	0.111	0.169	0.866
B x 8h	-0.976	0.331	-0.822	0.413	-1.760	0.081	-0.968	0.335
B x 24h	-0.350	0.727	0.039	0.969	-0.577	0.565	0.172	0.864
B x 48h	-1.042	0.300	-1.086	0.280	-2.353	***0.020**	-2.049	***0.043**

Planned comparisons II (Sampling time x Treatment)

	F	**p-value**	-	**F**	**p-value**
Ct x Ka: 0h	1.34	0.250	-	11.49	***0.001**
Ct x Ka: 0.5h	10.18	***0.002**	-	0.84	0.362
Ct x Ka: 1h	0.04	0.850	-	0.34	0.558
Ct x Ka: 2h	0.00	0.979	-	0.06	0.807
Ct x Ka: 4h	0.01	0.908	-	2.84	0.095
Ct x Ka: 8h	0.02	0.881	-	0.45	0.505
Ct x Ka: 24h	0.07	0.795	-	0.61	0.435
Ct x Ka: 48h	0.00	0.966	-	0.09	0.765

1.2. Effects of Kainic acid on C-FOS and BDNF protein levels in the telencephalon

Regarding C-FOS, (Figure 9) there was no effect of time on C-FOS protein levels (main effect of sampling time: ($F_{(8,83)}$=1.128; p=0.353). No significant differences were found between either the KA or the control group at any of the sampling points and the baseline (Table 3).

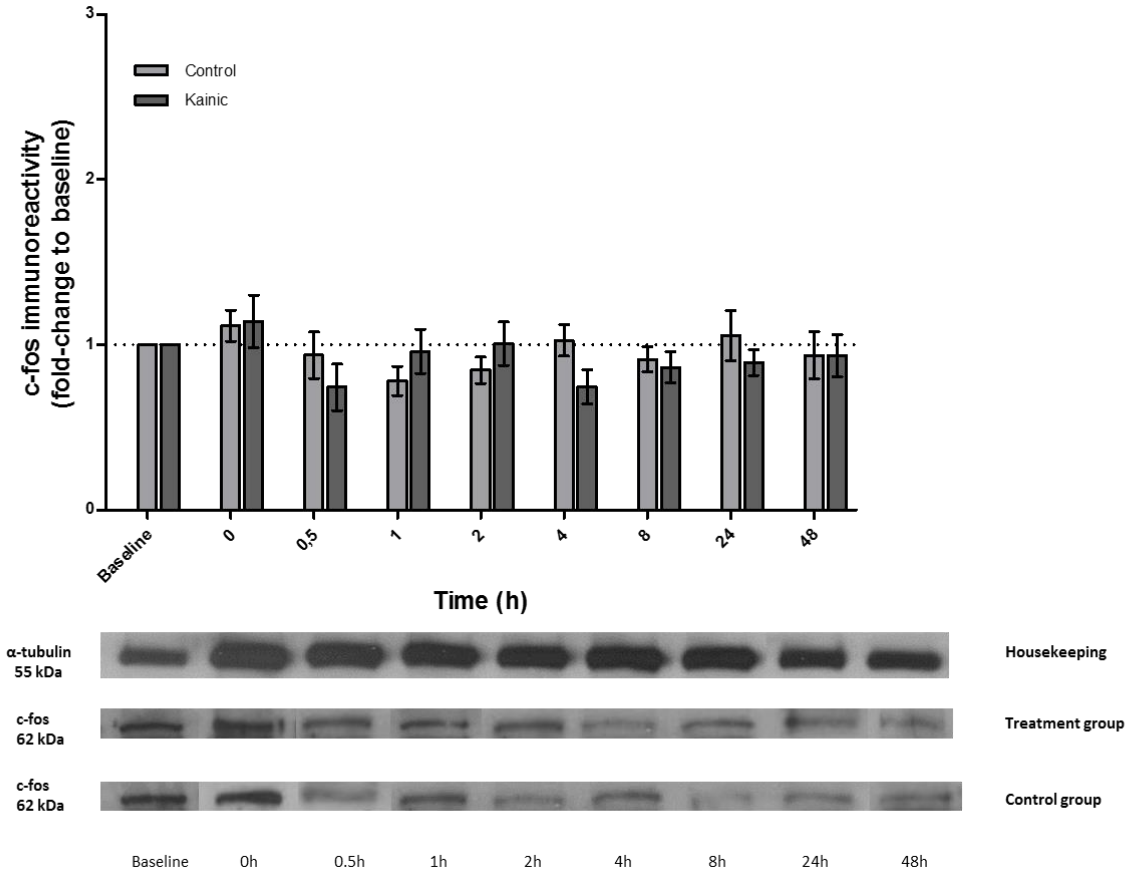

Figure 9 | Temporal changes in c-fos protein levels after a treatment with kainic acid. Animals were injected with either saline or kainic acid solution and sacrificed immediately, 30 min., 1hr, 2hr, 4hr, 8hr, 24hr or 48hr after injections. The time course, after either saline or KA administration, for increases in protein levels in telencephalon are shown for c-fos. Acute intraperitoneal kainic acid (0.5mg/Kg) does not induces an increase in protein levels. The results are the mean±SEM of seventeen different experiments performed in independent animals. *p≤0.05; **p≤0.01; ***p≤0.001

The temporal changes in pro-BDNF after a treatment with KA are represented in figure 10A. There was an effect of treatment on pro-BNDF levels ($F_{(2,100)}$=4.350;p=0.015)). There were differences between KA and control groups at the 1h and 48h post-injection (Table 3). In the

control group pro-BDNF levels were significantly higher than the baseline at 2h and 8h post-injection (Table 3). In the KA group pro-BDNF levels were higher than baseline at 0h; 1h; 24h and 48h post injection (Table 3).

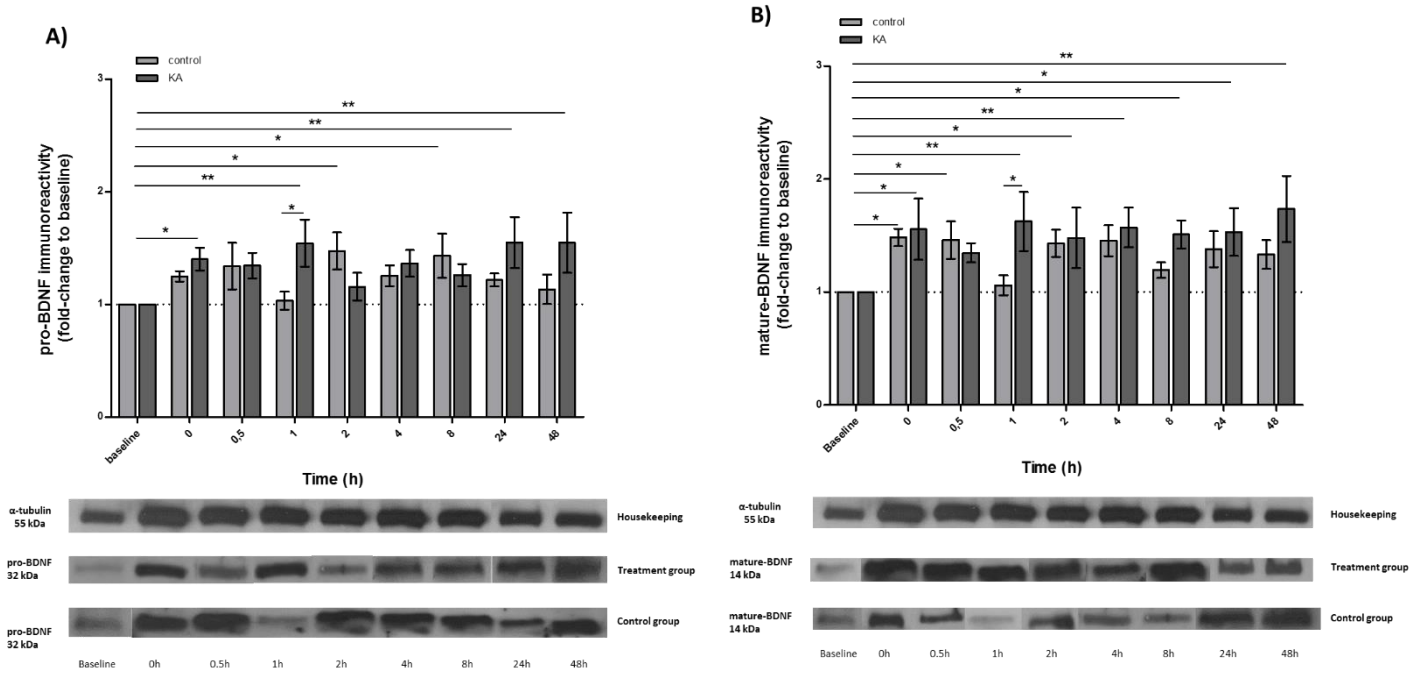

Figure 10 | Temporal changes in BDNF protein levels after treatment with kainic acid: A) pro-BDNF immunoreactivity; B) mature-BDNF immunoreactivity. Animals were injected with either saline or kainic acid solution and sacrificed immediately, 30 min., 1hr, 2hr, 4hr, 8hr, 24hr or 48hr after injections. The time course, after either saline or KA administration, for increases in protein levels in telencephalon are shown for BDNF. Acute intraperitoneal kainic acid (0.5mg/Kg) induces an increase in pro-BDNF levels 0hrs, 1hrs, 24hrs and 48hrs after administration; following an increase in mature-BDNF levels at 0hrs, 1hrs, 2hrs, 4hrs, 8hrs, 24hrs and 48hrs. The results are the mean±SEM of seventeen different experiments performed in independent animals. *p≤0.05; **p≤0.01; ***p≤0.001

Figure 10B shows the changes in mature-BDNF levels after treatment. There was a main effect of treatment on mature-BDNF levels ($F_{(2,99)}$=6.800; p=0.002)). In the control group, a significant increase in mature-BDNF is observed at 0h and 0.5h after administration of the saline solution. The KA group showed a significant increase of mature-BDNF levels at 0h; 1h; 2h; 4h; 8h 24h and 48h after administration of kainic acid (Table 3). Comparisons between the control and the KA group along sampling times, only showed a significant difference between these two groups at 1h post-administration ($F_{(1,79)}$=5.65; p=0.020).

Table 3 | ANOVA analyses for c-fos gene expression after a treatment with kainic acid (Ka) or saline solution (Ct) at different sampling timepoints (0h, 0.5h, 1h, 2h, 4h, 8h, 24h and 48h). Main effects and results from planned comparisons are present in this table. Abbreviations: B, baseline; Ct, control group; Ka, Kainic acid group. *p<0.005.

	C-FOS		pro-BDNF		mature-BDNF	
	F	***p-value***	**F**	***p-value***	**F**	***p-value***
Sampling time	1.128	0.353	0.749	0.648	1.307	0.250
Treatment	0.312	0.733	4.350	***0.015**	6.800	***0.002**
Sampling time x Treatment	1.021	0.446	1.442	0.142	1.339	0.194

Planned comparisons I (Baseline x Sampling time x Treatment)

	Control		KA		Control		KA		Control		KA	
	t-value	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**
B_x 0h	0.733	0.466	0.949	0.346	1.263	0.210	2.031	***0.045**	1.963	***0.053**	2.376	***0.020**
B_x 0.5h	-0.421	0.675	-1.633	0.107	1.718	0.089	1.737	0.086	1.968	***0.052**	1.487	0.141
B_x 1h	-1.467	0.147	-0.258	0.797	0.179	0.858	2.607	***0.011**	0.266	0.791	2.538	***0.013**
B_x 2h	-0.916	0.363	0.044	0.965	2.392	***0.019**	0.795	0.429	1.843	0.069	2.050	***0.043**
B_x 4h	0.164	0.870	-1.615	0.111	1.215	0.228	1.918	***0.058**	1.847	0.068	2.543	***0.013**
B_x 8h	-0.565	0.574	-0.870	0.387	2.183	***0.032**	1.315	0.192	0.831	0.408	2.179	***0.032**
B_x 24h	0.367	0.715	-0.710	0.480	1.096	0.276	2.775	***0.007**	1.623	0.108	2.269	***0.026**
B_x 48h	-0.384	0.702	-0.441	0.661	0.680	0.498	2.767	***0.007**	1.418	0.160	3.144	***0.002**

Planned comparisons II (Sampling time x Treatment)

	C-FOS		pro-BDNF		mature-BDNF	
	F	**p-value**	**F**	**p-value**	**F**	**p-value**
Ct x Ka: 0h	0.14	0.713	0.51	0.477	0.11	0.745
Ct x Ka: 0.5h	1.10	0.298	0.00	0.986	0.20	0.656
Ct x Ka: 1h	1.26	0.265	6.02	***0.016**	5.65	***0.02**
Ct x Ka: 2h	0.86	0.357	2.20	0.142	0.04	0.848
Ct x Ka: 4h	2.50	0.119	0.24	0.627	0.30	0.583
Ct x Ka: 8h	0.07	0.787	0.65	0.422	1.57	0.214
Ct x Ka: 24h	0.85	0.360	2.43	0.123	0.36	0.550
Ct x Ka: 48h	0.00	0.999	3.76	***0.056**	2.57	0.113

2.1. Object recognition test

Figure 11 shows the performance of zebrafish in the object recognition test. During the first day, animals did not exhibit preference between the two novel objects presented ($t_{(40)}$=0.235; *p*=0.815). On the second day, animals did not exhibit a significant measurable preference for a novel object ($t_{(40)}$=0.264; *p*=0.793) (Figure 11-A). The focal fish explore more often the objects on the first day, showing a significant reduction on exploration time on the second day ($t_{(40)}$=2.465; *p*=0.018) (Figure 11-B).

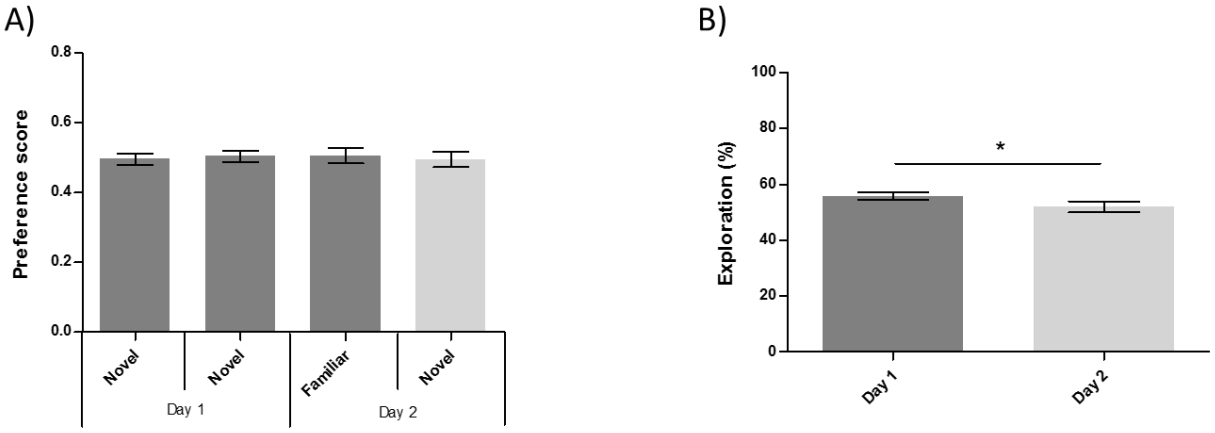

Figure 11| Object recognition. The time spent in each chamber were used to investigate object recognition. On day 1, animals were freely to explore two naive objects different in shape but with the same volume and color. 24hr later, individuals were faced with a familiar and a non-familiar object. Focal fish cannot discriminate between a familiar and a non-familiar object. A) Preference score and B) exploration time (%) *p≤0.05

2.2. Effects of different sensory modalities in social recognition using the social discrimination test

Zebrafish failed to discriminate between novel and familiar conspecifics when only chemical cues are available (Figures 12-A). As expected on the first day of these tests (i.e. chemical-only) focal fish spent similar amounts of time exploring each individual, indicating no significant preference for any of them (chemical-only: ($t_{(41)}$ =0.455; *p*=0.651, Fig. 12-A). Contrary to the predictions, 24h after the exposure to conspecific chemical cues, focal fish also spent similar amounts of time close to the familiar and to the novel stimulus fish (chemical-only: $t_{(41)}$=0.092; *p*=0.927).

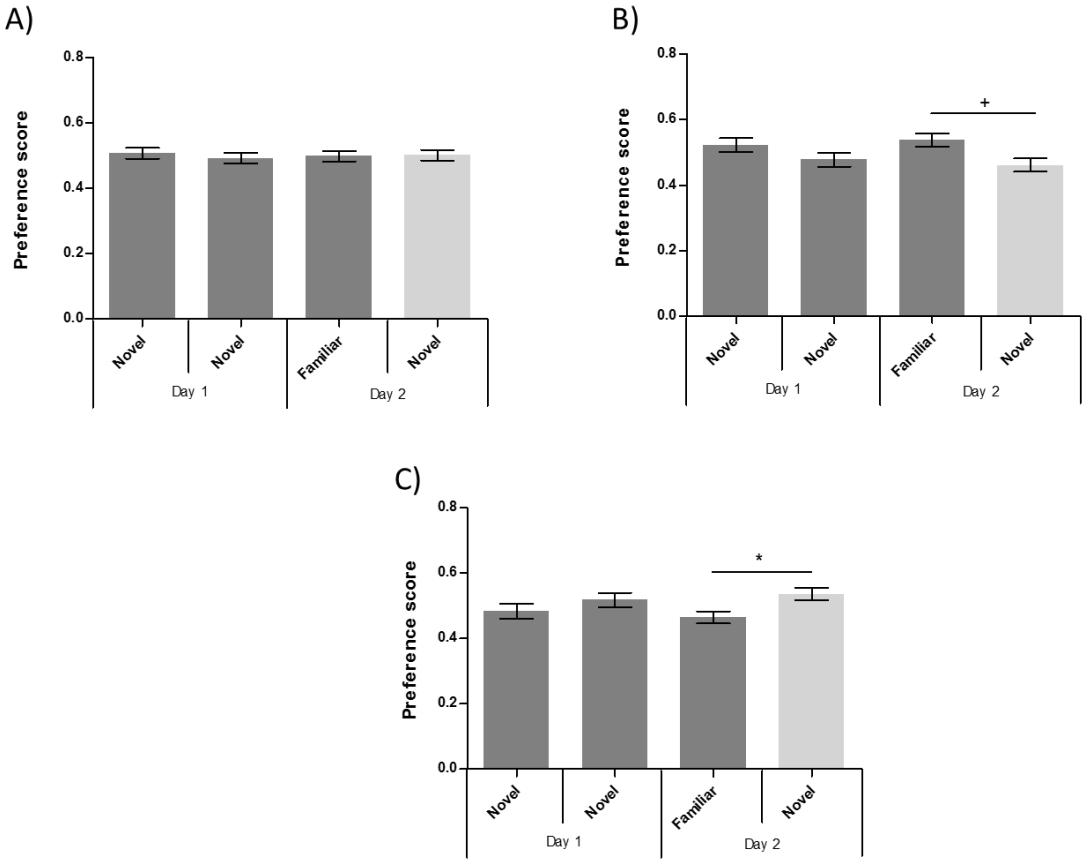

Figure 12 | Social memory assessment for olfactory (A), visual (B) and integration of both (C) stimuli. The time spent in each chamber were used to investigate social recognition, based on different types of sensorial modalities. On day 1, animals were freely to explore two naive conspecifics. 24hr later, individuals were faced with a familiar and a non-familiar conspecifics. A) Focal fish cannot discriminate familiar from non-familiar conspecifics only based on olfactory interaction; B) Focal fish discriminate familiar from non-familiar conspecifics only based on visual interaction; C) Focal fish discriminate familiar from non-familiar conspecifics based on visual and olfactory interactions. $^{+}p<0.06$; $*p\leq0.05$

When visual-only or both visual and chemical conspecific cues are accessible, zebrafish discriminate between a familiar and a novel conspecific 24 h after the first exposure to the familiar conspecific. As predicted, on day 1 focal fish shows no preference between the two novel individuals (visual-only: $t_{(40)}$ = 1.035; p=0.307, Fig.12-B; multimodal: $t_{(40)}$ = 0.749; p=0.458, Fig.12-C).However, on day 2 focal fish shows preference for the novel fish (multimodal: $t_{(40)}$ = 2.052; p=0.047, Fig.12-C) or for the familiar fish (visual-only: $t_{(40)}$ = 1.924; p=0.061, Fig.12-B).

Zebrafish also explore more often conspecifics on the first day. On the second day, they reduces significantly exploration towards individuals (chemical-only: $t_{(41)}$ = 3.121; p=0.003 ; visual-only: $t_{(40)}$ = 7.696; p=0.000; multimodal: $t_{(40)}$ = 6.791; p=0.000) (Figure 13 A-C).

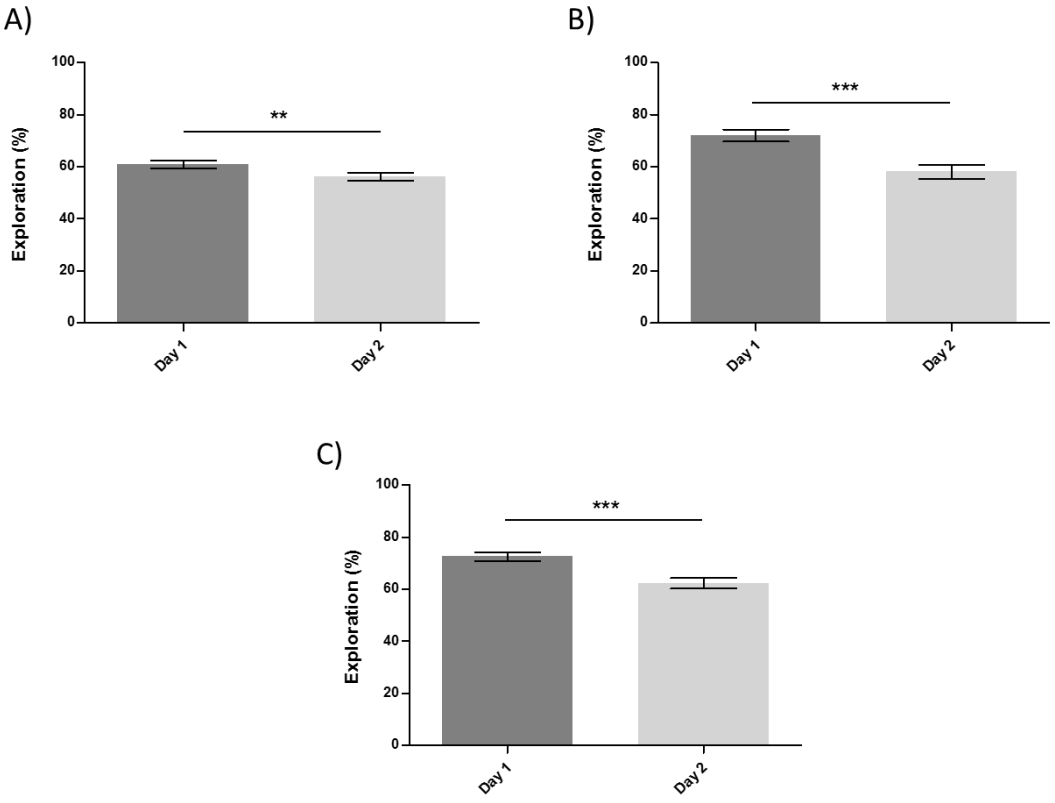

Figure 13 |Stimulus exploration (%) for social discrimination memory using olfactory (A), visual (B) and multimodal (C) cues. On the first day animals explore actively the conspecifics. On the second day individuals reduce significantly the exploration time.

2.3. Test of social recognition using the habituation-dishabituation paradigm

Figure 14 shows the performance of zebrafish in the habituation-dishabituation paradigm. In the dishabituation phase of this paradigm, animals were faced with different conspecifics on day 1 and day 2. As a control, in the habituation phase, animals were faced with the same conspecific on day 1 and day 2. The time point tested showed no significant reductions in the duration of investigation time between the two paradigms (N+F vs N+N) ($t_{(42)}$=0.8142; p=0.4202; Fig.14). These results indicate that zebrafish failed to recognize familiar conspecifics using this paradigm.

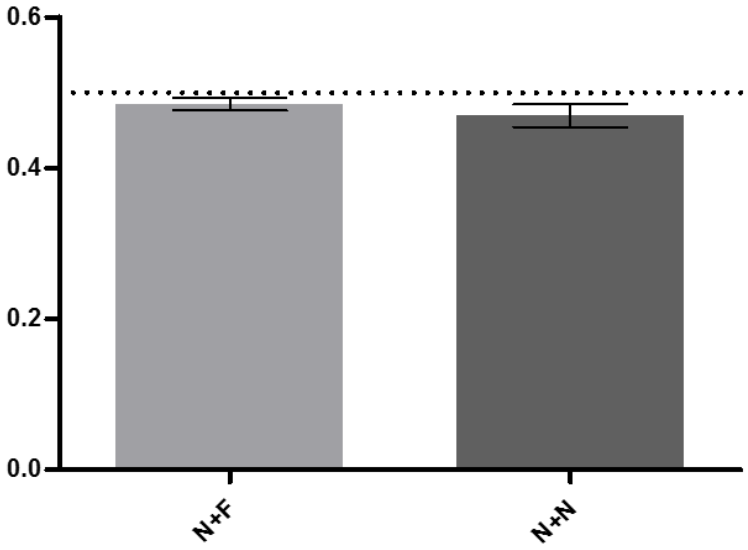

Figure 14 | Social memory assessment using a habituation-dishabituation paradigm. The time spent in each chamber were used to investigate social recognition. On day 1, animals were freely to explore one naive conspecific. 24hr later, individuals were faced with a familiar (N+F) or a novel conspecific (N+N).

2.4. Sociability and memory performance

The results show a positive correlation between the social memory test (V+C) and sociability (Pearson's r = 0.3658; R^2 = 0.1338; *p*=0.026), indicating that social individuals have a better ability to discriminate and recognize conspecifics.

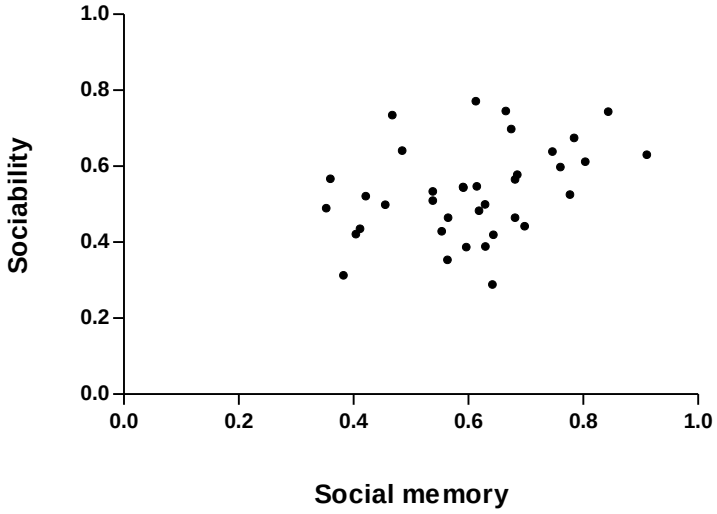

Figure 15 |Correlation between social memory performance and sociability.

2.5. Effects of Social memory on bdnf gene expression in the hippocampus and amygdala

The effect of the different memory tests on *bdnf* gene expression in two telencephalic regions (Dm and Dl) is presented in Fig. 16. There were no significant differences in *bdnf* gene expression between tests in Dm, a putative homologue of the mammalian amygdala ($F_{(5,29)}$=1.762; p=0.152) (Table 4).

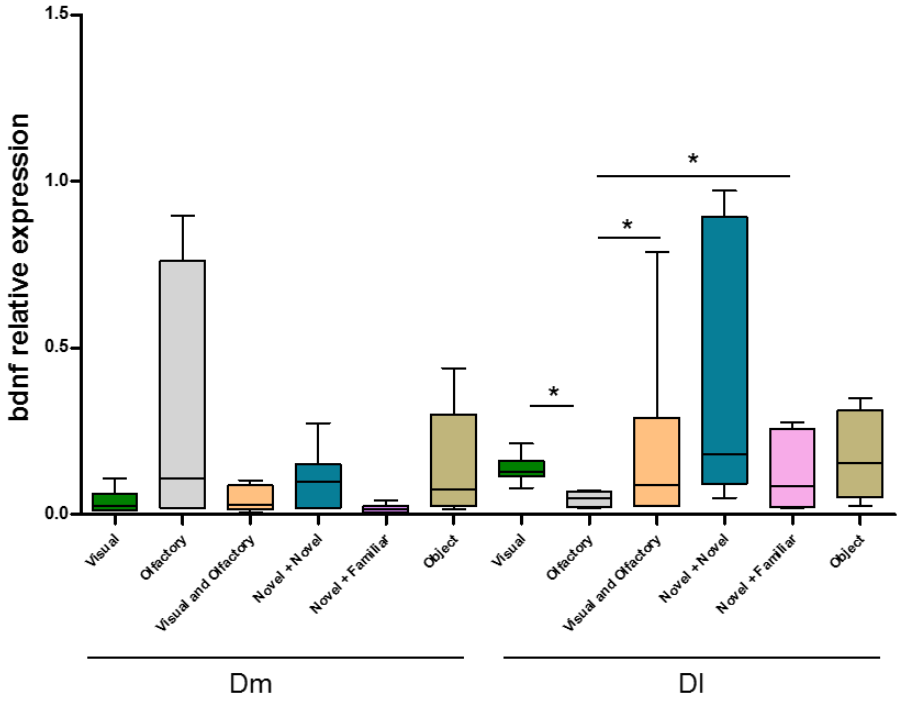

Figure 16 |*bdnf* gene expression changes after memory tests. *p≤0.05

In contrast, *bdnf* gene expression was significantly different between memory tests in Dl, a region that is a teleost homologous of the mammalian hippocampus ($F_{(5,32)}$=2.661; p=0.040). However, there were no significant differences between successful vs. unsuccessful memory tests (e.g. visual + chemical social recognition vs. object recognition; Table 4).

Table 4 | Results determined by ANOVA followed by Tukey's post-hoc test.

	bdnf	
	Dm	**Dl**
	p-value	
V x C	0.988	***0.051**
V x V+C	0.998	1
V x N+N	0.251	0.984
V x N+F	1	1
V x O	0.999	0.845
C x V+C	0.909	***0.051**
C x N+N	0.122	0.229
C x N+F	0.994	***0.039**
C x O	0.930	0.368
V+C x N+N	0.463	0.991
V+C x N+F	0.994	0.999
V+C x O	1	0.871
N+N x N+F	0.212	0.945
N+N x O	0.328	0.997
N+F x O	0.998	0.716

CHAPTER 5

Discussion

We initially examined gene expression of *c-fos* and *bdnf* at several time points (0hr, 0.5hr, 1hr, 2hr, 4hr, 8hr, 24hr and 48hr) after a kainic acid treatment to determine the time course over which they were regulated. This is important to this work in order to establish *c-fos* and bdnf as immediate early genes in zebrafish and to determine the necessary delay between stimulation and sacrifice of animals after behavioral tests. We found that *c-fos* showed a transient up-regulation 30 min after injection with kainic acid and after 1 hour, expression levels were back to baseline levels. This down-regulation pattern was observed during the subsequent 48hrs. Kainic acid is an analogue of the excitatory neurotransmitter glutamate and it induces convulsions which recapitulate both electrophysiological and behavioral components of epilepsy (Blendy et al., 1995). In mammalian cells, it promotes a rapid induction of IEG's, specially BDNF (Binder et al., 2001). Several studies have shown that KA treatment induces *c-fos* expression, with particular accumulation in the hippocampal formation. Our results are in agreement with previous findings that show that IEG's have a peak of expression 30 minutes after stimulation (Bisler et al., 2002; Burmeister & Fernald, 2005; Mello & Ribeiro, 1998).

Regarding *bdnf*, an increase in *bdnf* mRNA levels was observed 0hrs after administration of saline solution with a decrease after 48 hours. These results can lead to two possible explanatory hypotheses. A first explanation can be that the peak of rapid *bdnf* induction occurred before our first sampling point (30 min), and we missed it. If this was the case, kainic acid promotes an instantaneous induction of *bdnf* between 0 and 30 minutes. A second explanation can be justified by the sampled brain area. The zebrafish telencephalon includes different anatomic and functional subdomains such as: Dl (lateral zone of dorsal telencephalic area), Dm (medial zone of dorsal telencephalic area, Dp (posterior zone of dorsal telencephalic area, Dc (central zone of dorsal telencephalic area, Vv (ventral nucleus of ventral telencephalic area), Vd (dorsal nucleus of ventral telencephalic area) and Vc (central nucleus of ventral telencephalic area). In this study, the telencephalon was sampled as a whole including all these subdomains which may have different time courses of response to kainic acid stimulation.

In the present study, zebrafish exhibited consistent recognition of a previously encountered (familiar) conspecific after 24 h. This result confirms previous reports using mouse models (Kogan et al., 2000) and extends these findings to this teleost fish. A recent study from Barba-Escobedo and Gould (2012) reported short-term social recognition memory in zebrafish

(Barba-Escobedo & Gould, 2012); here we show, for the first time, long-term social recognition memory in this animal model.

We also presented evidence that in zebrafish the formation of long-term social recognition memory requires use of both chemical and visual cues. Chemical signaling is essential in the aquatic environment. Water acts like as a solvent and as a medium to disperse cues. The use of chemical cues may be particularly useful when visual communication is limited, e.g. darkness, deep or turbid water as well as in highly structured environments. Chemical communication uses urine, mucus and faeces to convey information about age, sex and dominance that mediates several social interactions between fish, including mate-choice, dominance relationships and predator recognition (Ward et al., 2004). A general form of recognition based on odor cues has been documented in fathead minnows and sticklebacks (Brown & Smith, 1994). However, our results suggest that animals spend the same proportion of time exploring familiar and unfamiliar conspecific chemical cues (Figure 11B). The odor of an individual is strongly influenced by both recent habitat use and diet (Ward, Hart, & Krause, 2004), that mediates association preferences suggesting a chemical self-referencing (Mateo, 2004). However, in laboratory conditions, the use of chemical cues is conditioned to particular water chemistry conditions and to specific diet - in contrast to the natural environment, where fish are free to move around between different microhabitats and feed widely. In the current study, the laboratory conditions could have the effect of minimizing any differences between the chemical cues that individuals produce. In guppies (*Poecilia reticulate)*, the recognition of particular individuals seems to be based in a more specific type of recognition that requires the use of visual cues (Griffiths & Magurran, 1997). In this study, zebrafish expressed a marginally non-significant preference for the visual cues of individuals with whom they had had prior contact. Individuals were observed more often on the side with visual cues of familiar conspecifics compared with non-familiar conspecifics.

In the current study, two mechanisms (chemical and visual) seem to operate in conjunction to allow discrimination of conspecifics. Taken as a whole, our findings suggest that in zebrafish, social recognition is achieved by using a combination of visual and chemical cues that are used as self-referent matching. This observation also suggests that long-term social memory requires a multimodal encryption in zebrafish. Some neurobiological evidences of this remarkable ability in zebrafish can justify the use of these two sensory cues. In zebrafish, the terminal nerve (TN, aka nucleus olfactoretinalis) is located in the olfactory bulbs and project to the telencephalon,

the tectal targets and to the contralateral retina (Maaswinkel, 2003). This pathway receives synaptic inputs from the olfactory bulbs and seems to be involved in processing sensory information of other modalities such as visual and somatosensory information (Yamamoto & Ito, 2000). Apparently, TN receives inputs to the retina upon olfactory stimulation, which has an effect on behavioral visual sensitivity (Maaswinkel, 2003). Thus, rather than being a sensory nerve, the TN is responsible for olfacto-retinal modulation. In this study, we suggest that zebrafish needs to integrate the two sensory systems to discriminate between two conspecifics, and this integration may be explained by the TN pathway.

Our results also show that individuals prefer new individuals rather than familiar ones (Figure 11C). This result can be viewed as unexpected, since zebrafish form structured shoals and prefer shoaling with familiar relatives (Engeszer et al., 2004). However, shoaling preferences are based on learned social preferences such as appearance, kinship or current behavior of individuals (Engeszer, Ryan, & Parichy, 2004b; OlsÉn et al., 1998) and should be distinguished from familiarity preferences. In some cases, fish could avoid familiar individuals instead preferring unfamiliar ones (Barba-Escobedo & Gould, 2012), a preference that can maximize mating opportunities for males and improve fitness.

We used two versions of social memory paradigms: a discrimination paradigm (Engelmann et al., 1995) and a habituation-dishabituation paradigm (Thor & Holloway, 1982), both adapted from mouse studies. Our results showed that different paradigms could produce different results. The discrimination paradigm showed robust formation of long-term social memory, whereas the habituation-dishabituation test failed to demonstrate social memory formation (Figure 12). Even though it is less popular and less utilized than the habituation/dishabituation procedure, evidences demonstrate that the social discrimination paradigm seems to be a more sensitive test of assessing social recognition, because it has allowed the emergence of social discrimination in animals that appeared to possess no social recognition abilities when tested in the habituation/dishabituation paradigm (Choleris et al., 2006; Engelmann et al., 1995). Another possibility is that repeated testing of the same individual led to non-specific behavioral changes, as sensitization to the habituation-dishabituation procedure. These effects may mask, upon dishabituation testing, specific individual recognition-related changes.

The novel object recognition test evaluates an animal's ability to distinguish between two different objects. Our results suggest that zebrafish were not able to discriminate between a

familiar and a novel object, after a single exposure, 24 hours after the test. In the test phase, no differences were found between the preference for a novel or a familiar object. There are at least two possible explanations for this result. One possible reason lies in the different characteristics of the stimuli to be discriminated. In this study, we used stimuli that differed only in a single cue (geometric form) while keeping other features (i.e. same color but different shapes), which made the task quite difficult to perform. A second reason is related to the way in which zebrafish explore the stimuli, which may differ consistently to what is considered object exploration in other species. In other species, they show manipulatory behavior, chewing and sniffing (Heyser & Chemero, 2012). In zebrafish, object exploration can be described as swimming near the stimuli and/or staying oriented towards the stimuli, which can produce difficult behavioral phenotypes to evaluate.

The behavioral paradigms also show that zebrafish explore more often the stimuli fish on the first day. On the second day, animals reduce significantly the exploratory behavior. An explanation of this result can be test habituation. The exploration decrease observed on the second day suggests that animals remember the behavioral task that they did previously. This result implies that zebrafish can recall behavioral tasks for, at least, 24 hours.

As expected, the sociability test indicates that zebrafish preferred other fish to being alone, showing that they are social animals. The correlation between the sociability test and the social memory test suggests that more social individuals have a better ability to recognize and remember conspecifics. This finding support the idea – initially tested in humans – that performance on different cognitive tasks shows robust positive correlations (Neisser et al., 1996; Plomin, 2001). Recently, few studies have shown that animals that perform better in one cognitive task are sexually preferred, because they are better at cognitive tasks in general (Keagy et al., 2009; Shohet & Watt, 2009). In these study, we support previous findings in this field and demonstrate that more social individuals, have more ability to remember and identify conspecifics.

The current study also sought to examine whether social and asocial memory paradigms induced modifications in the expression of *bdnf* mRNA, and in which major brain regions it occurs. The relationship between behavioral performance and local levels *bdnf* mRNA in the hippocampal and amygdala sub-regions was assessed 2 hours after the behavioral tasks. In the Dm (homolog to amygdala) no significant differences were found in mRNA *bdnf* levels, after the different

behavioral tasks. On the other hand, the Dl (homolog to hippocampus) showed significant differences in *bdnf* mRNA levels, dependent on behavioral task. Animals that performed the social memory paradigms based only on olfactory cues, showed relative low-levels of *bdnf* gene compared with animals that performed the same paradigm based on only visual cues or on both visual and olfactory cues. This result suggests that the integration of both visual and olfactory cues activates endogenous neurotrophin signaling in the hippocampus, providing more neurotrophic support for the Dl neurons. The subsequent plastic changes, caused by an up-regulation of *bdnf*, could modulate cellular modification of neural networks that contributes to social memory (Bekinschtein et al., 2013). The *bdnf* low-levels (olfactory-only test) could be explained based on previous works that showed that olfactory learning tests caused no changes in synaptic activity in the hippocampus (Naimark et al., 2007). In the hippocampus *bdnf* mRNA increases with hippocampal-dependent tasks (Mizuno et al., 2000). However, few studies have evaluated the contribution of different sensory systems to memory formation. Broad and colleagues (2002), showed that the formation of mouse recognition memory was associated with low-doses of *bdnf* mRNA expression in the olfactory bulbs (Broad et al., 2002).

Taken together, these finding suggests that BDNF is not directly involved in the regulation of social memory in the amygdala, contrary with what happen in the hippocampus. Several studies have reported that in the amygdala, oxytocin and vasopressin are the neuropeptides responsible for the regulation of social memory (Choleris et al., 2007; Ferguson et al., 2001). It has been demonstrated that, in zebrafish, connections between Dl (hippocampus homolog) and Dm (amygdala homolog) divisions may bear an important intrinsic physiological role in synaptic function (Ng et al., 2012). These connections are mediated by glutamatergic neurotransmission, where the Dl region induces a robust and stable LTP at the Dm division (Ng et al., 2012). Here we propose that the high levels of BDNF observed in the Dl could affect LTP and consequently the secretion of OT in the Dm. Our findings support a new role for the neural connections between the Dl and Dm regions, mediated by BDNF, in the mechanism of social memory.

CHAPTER 6

Conclusions

Experiment 1 suggests that an external stimulus elicits an increment of *c-fos* gene expression levels, after 30 minutes. After 30 minutes, this up-regulation is followed by an abrupt decrease in *c-fos* levels that remains for 48 hours. However, C-FOS protein levels did not show significant differences along the timeline and between treatments (control vs treatment). Regarding *bdnf,* this gene shows an up-regulation 0 minutes after administration of a saline solution. 48 hours after administration, both treatments (control and treatment) decrease *bdnf* expressions levels. At protein level, pro-BDNF shows an up-regulation 2h and 8h after saline solution administration and 0min., 1h, 4h, 24h e 48h after treatment with kainic acid; mature-BDNF shows an up-regulation 0 min. and 30 min. after saline solution administration; and 0min., 1h, 2h, 4h, 8h, 24h and 48h after kainic solution administration.

Experiment 2 shows that zebrafish can form robust long-term social recognition memory, using a discrimination paradigm. This type of recognition requires a multimodal sensorial system (i.e. based on olfactory and visual cues), which is essential for recognition. When animals use only chemical cues, they cannot recognize individuals 24 hours after the first encounter. However, when visual cues are present they seem to prefer to explore familiar animals. Although the preference score in these two tests is different, in both tasks encompassing visual cues they seem to recognize individuals. The habituation-dishabituation paradigm failed to demonstrate social recognition in zebrafish. The object recognition test failed to demonstrate that individuals recognize different objects 24 hours after being familiarized with them. Zebrafish demonstrate a highly exploratory behavior, and spent on average 60% of total task time exploring the stimuli. They are also able to remember the behavioral paradigm for at least 24h.

We suggest that *bdnf* is involved in social memory and it's expression is regulated in the hippocampus by different sensory systems. The recognition of conspecifics, when using visual or both visual and chemical, is accomplished by the presence of high-doses of *bdnf* in the hippocampus. In contrast, when individuals use only chemical cue information, the hippocampus shows low-doses of *bdnf*. We propose that BDNF high levels observed in the Dl could affect LTP and consequently the production and secretion of OT in the Dm. We propose that long-term social memory requires a multimodal encryption in zebrafish with sensorial cues displaying different weights in social memory formation. Chemical cues are not enough to trigger the formation of social memory; whereas visual cues are. In addition, visual cues combined with chemicals cues provide the most robust trigger to the development of long-term social memory, which seems to

be largely mediated by the up-regulation of bdnf levels in the Dl. We also propose that high levels of BDNF observed in the Dl will lead to LTP and increase the strength of the connections between the Dl and the Dm, ultimately leading to the secretion of the OT in the Dm. In turn, the secretion of OT in the Dm will allow zebrafish to recognize familiar conspecifics and is a crucial determinant of the social abilities of zebrafish.

REFERENCES

Adolphs, R. (2010). Conceptual challenges and directions for social neuroscience. *Neuron*, *65*(6), 752–67.

Alberini, C. M., Ghirardi, M., Huang, Y. Y., Nguyen, P. V, & Kandel, E. R. (1995). A molecular switch for the consolidation of long-term memory: cAMP-inducible gene expression. *Annals of the New York Academy of Sciences*, *758*, 261–86.

Aoki, T., Kinoshita, M., Aoki, R., Agetsuma, M., Aizawa, H., Yamazaki, M., Okamoto, H. (2013). Imaging of Neural Ensemble for the Retrieval of a Learned Behavioral Program. *Neuron*, *78*(5), 881–894.

Avdesh, A., Chen, M., Martin-iverson, M. T., Verdile, G., Mondal, A., & Martins, R. N. (2010). Natural Colour Preference in the Zebrafish (Danio rerio), Proceedings of measuring behavior, 155–157.

Barba-Escobedo, P. A., & Gould, G. G. (2012). Visual social preferences of lone zebrafish in a novel environment: strain and anxiolytic effects. *Genes, Brain, and Behavior*, *11*(3), 366–73.

Barcellos, L. J. G., Ritter, F., Kreutz, L. C., Quevedo, R. M., da Silva, L. B., Bedin, A. C., Cericato, L. (2007). Whole-body cortisol increases after direct and visual contact with a predator in zebrafish, Danio rerio. *Aquaculture*, *272*(1-4), 774–778.

Beason-Held, L. L., Rosene, D. L., Killiany, R. J., & Moss, M. B. (1999). Hippocampal formation lesions produce memory impairment in the rhesus monkey. *Hippocampus*, *9*(5), 562–74.

Bekinschtein, P., Cammarota, M., & Medina, J. H. (2013). BDNF and memory processing. *Neuropharmacology*, *5*, 1–7.

Benelli, A., Bertolini, A., Poggioli, R., Menozzi, B., Basaglia, R., & Arletti, R. (1995). Polymodal dose-response curve for oxytocin in the social recognition test. *Neuropeptides*, *28*(4), 251–255.

Bilotta, J., Risner, M. L., Davis, E. C., & Haggbloom, S. J. (2005). Assessing appetitive choice discrimination learning in zebrafish. *Zebrafish*, *2*(4), 259–68.

Binder, D. K., Croll, S. D., Gall, C. M., & Scharfman, H. E. (2001). BDNF and epilepsy: too much of a good thing? *Trends in Neurosciences*, *24*(1), 47–53.

Bird, C. M., & Burgess, N. (2008). The hippocampus and memory: insights from spatial processing. *Nature Reviews. Neuroscience*, *9*(3), 182–94.

Bisler, S., Schleicher, A., Gass, P., Stehle, J. H., Zilles, K., & Staiger, J. F. (2002). Expression of c-Fos, ICER, Krox-24 and JunB in the whisker-to-barrel pathway of rats: time course of induction

upon whisker stimulation by tactile exploration of an enriched environment. *Journal of Chemical Neuroanatomy*, *23*(3), 187–98.

Blank, M., Guerim, L. D., Cordeiro, R. F., & Vianna, M. R. M. (2009). A one-trial inhibitory avoidance task to zebrafish: rapid acquisition of an NMDA-dependent long-term memory. *Neurobiology of Learning and Memory*, *92*(4), 529–34.

Blaser, R. E., & Rosemberg, D. B. (2012). Measures of anxiety in zebrafish (Danio rerio): Dissociation of black/white preference and novel tank test. *PLoS ONE*, *7*(5), 1–8.

Blendy, J. A., Schmid, W., Kiessling, M., & Schlitz, G. (1995). Effects of kainic acid induced seizures on immediate early gene expression in mice with a targeted mutation of the CREB gene. *Brain Research*, *681*, 8–14.

Bliss, T. V, & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *The Journal of Physiology*, *232*(2), 331–56.

Braida, D., Ponzoni, L., Martucci, R., & Sala, M. (2014). A new model to study visual attention in zebrafish. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *55*, 80–6.

Braubach, O. R., Wood, H.-D., Gadbois, S., Fine, A., & Croll, R. P. (2009). Olfactory conditioning in the zebrafish (Danio rerio). *Behavioural Brain Research*, *198*(1), 190–8.

Brown, G. E., & Smith, R. J. (1994). Fathead minnows use chemical cues to discriminate natural shoalmates from unfamiliar conspecifics. *Journal of Chemical Ecology*, *20*(12), 3051–61.

Burmeister, S. S., & Fernald, R. D. (2005). Evolutionary conservation of the egr-1 immediate-early gene response in a teleost. *The Journal of Comparative Neurology*, *481*(2), 220–32.

Carr, W. J., Yee, L., Gable, D., & Marasco, E. (1976). Olfactory recognition of conspecifics by domestic Norway rats. *Journal of Comparative and Physiological Psychology*, *90*(9), 821–8.

Choleris, E., Little, S. R., Mong, J. A., Puram, S. V, Langer, R., & Pfaff, D. W. (2007). Microparticle-based delivery of oxytocin receptor antisense DNA in the medial amygdala blocks social recognition in female mice. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(11), 4670–5.

Choleris, E., Ogawa, S., Kavaliers, M., Gustafsson, J. Å., Korach, K. S., Muglia, L. J., & Pfaff, D. W. (2006). Involvement of estrogen receptor α, β and oxytocin in social discrimination: A detailed behavioral analysis with knockout female mice. *Genes, Brain and Behavior*, *5*(7), 528–539.

Citri, A., & Malenka, R. C. (2008). Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *33*(1), 18–41.

Cognato, G. D. P., Bortolotto, J. W., Blazina, A. R., Christoff, R. R., Lara, D. R., Vianna, M. R., & Bonan, C. D. (2012). Y-Maze memory task in zebrafish (Danio rerio): the role of glutamatergic and cholinergic systems on the acquisition and consolidation periods. *Neurobiology of Learning and Memory*, *98*(4), 321–8.

Colicos, M. a, & Syed, N. I. (2006). Neuronal networks and synaptic plasticity: understanding complex system dynamics by interfacing neurons with silicon technologies. *The Journal of Experimental Biology*, *209*(12), 2312–2319.

Colwill, R. M., Raymond, M. P., Ferreira, L., & Escudero, H. (2005). Visual discrimination learning in zebrafish (Danio rerio). *Behavioural Processes*, *70*(1), 19–31.

Connell, L. A. O., & Hofmann, H. A. (2011a). Genes , hormones , and circuits : An integrative approach to study the evolution of social behavior. *Frontiers in Neuroendocrinology*, *32*(3), 320–335.

Connell, L. A. O., & Hofmann, H. A. (2011b). The Vertebrate Mesolimbic Reward System and Social Behavior Network : A Comparative Synthesis, *3639*, 3599–3639.

Cowansage, K. K., LeDoux, J. E., & Monfils, M.-H. (2010). Brain-derived neurotrophic factor: a dynamic gatekeeper of neural plasticity. *Current Molecular Pharmacology*, *3*(1), 12–29.

Cunha, C., Brambilla, R., & Thomas, K. L. (2010). A simple role for BDNF in learning and memory ? 3, 1–14.

Dawkins, M. S. (1995). How Do Hens View Other Hens ? The Use of Lateral and Binocular Visual Fields in Social Recognition. *Behaviour*, *132*(7), 591–606.

Duckworth, R.A. (2009). The role of behavior in evolution: a search for mechanisms. *Evol. Ecol.* 23, 513–531.

Edelmann, E., Leßmann, V., & Brigadski, T. (2013). Pre- and postsynaptic twists in BDNF secretion and action in synaptic plasticity. *Neuropharmacology*.

Elgersma, Y., & Silva, a J. (1999). Molecular mechanisms of synaptic plasticity and memory. *Current Opinion in Neurobiology*, *9*(2), 209–13.

Engelmann, M., Ebner, K., Wotjak, C. T., & Landgraf, R. (1998). Endogenous oxytocin is involved in short-term olfactory memory in female rats. *Behavioural Brain Research*, *90*(1), 89–94.

Engelmann, M., Wotjak, C. T., & Landgraf, R. (1995). Social discrimination procedure: An alternative method to investigate juvenile recognition abilities in rats. *Physiology and Behavior*, *58*(2), 315–321.

Engeszer, R. E., Ryan, M. J., & Parichy, D. M. (2004). Learned social preference in zebrafish. *Current Biology*, *14*(10), 881–4.

Ferguson, J. N., Aldag, J. M., Insel, T. R., & Young, L. J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(20), 8278–8285.

Ferguson, J. N., Young, L. J., Hearn, E. F., Matzuk, M. M., Insel, T. R., & Winslow, J. T. (2000). Social amnesia in mice lacking the oxytocin gene. *Nature Genetics*, *25*(3), 284–288.

Ferguson, J. N., Young, L. J., & Insel, T. R. (2002). The neuroendocrine basis of social recognition. *Frontiers in Neuroendocrinology*, *23*(2), 200–24.

Gao, X.-M., Elmer, G. I., Adams-Huet, B., & Tamminga, C. A. (2009). Social memory in mice: disruption with an NMDA antagonist and attenuation with antipsychotic drugs. *Pharmacology, Biochemistry, and Behavior*, *92*(2), 236–42.

Gerlach, G., Hodgins-Davis, A., Avolio, C., & Schunter, C. (2008). Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proceedings. Biological Sciences / The Royal Society*, *275*(1647), 2165–70.

Gerlach, G., & Lysiak, N. (2006). Kin recognition and inbreeding avoidance in zebrafish, Danio rerio, is based on phenotype matching. *Animal Behaviour*, *71*(6), 1371–1377.

Gerlai, R., Lahav, M., Guo, S., & Rosenthal, A. (2000). Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. *Pharmacology, Biochemistry, and Behavior*, *67*(4), 773–82.

Germana, A., Laura, R., Montalbano, G., Guerrera, M., Amato, V., Zichichi, R., Vega, J. (2010). Expression of Brain-Derived Neurotrophic Factor and TrkB in the Lateral Line System of Zebrafish During Development. *Cell Mol Neurobiol*, *30*, 787–793.

Gheusi, G., Bluthé, R. M., Goodall, G., & Dantzer, R. (1994). Social and individual recognition in rodents: Methodological aspects and neurobiological bases. *Behavioural Processes*, *33*(1-2), 59–87.

Goelet, P., Castellucci, V. F., Schacher, S., & Kandel, E. R. (1986). The long and the short of long-term memory--a molecular framework. *Nature*, *322*(6078), 419–22.

Goodson, J. L., Evans, A. K., Lindberg, L., & Allen, C. D. (2005). Neuro-evolutionary patterning of sociality. *Proceedings. Biological Sciences / The Royal Society*, *272*(1560), 227–35.

Götz, R., Raulf, F., & Schartl, M. (1992). Brain-derived neurotrophic factor is more highly conserved in structure and function than nerve growth factor during vertebrate evolution. *Journal of Neurochemistry*, *59*(2), 432–42.

Griffiths, S. W., & Magurran, A. E. (1997). Schooling preferences for familiar fish vary with group size in a wild guppy population. *Proceedings of the Royal Society B: Biological Sciences*, *264*(1381), 547–551.

Guhl, A M and Ortman, L. L. (1953). Visual patterns in the recognition of individuals among chickens. *The Condor*, *55*(6), 287–298.

Hall, J., Thomas, K. L., & Everitt, B. J. (2000). Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nature Neuroscience*, *3*(6), 533–5.

Hashimoto, M., & Heinrich, G. (1997). Brain-derived neurotrophic factor gene expression in the developing zebrafish. *International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience*, *15*(8), 983–97.

Heyser, C. J., & Chemero, A. (2012). Novel object exploration in mice: Not all objects are created equal. *Behavioural Processes*, *89*(3), 232–238.

Hinz, C., Kobbenbring, S., Kress, S., Sigman, L., Müller, A., & Gerlach, G. (2013). Kin recognition in zebrafish, Danio rerio, is based on imprinting on olfactory and visual stimuli. *Animal Behaviour*, *85*(5), 925–930.

Hitti, F. L., & Siegelbaum, S. a. (2014). The hippocampal CA2 region is essential for social memory. *Nature*, *508*(7494), 88–92.

Hlinák, Z., & Krejcí, I. (2002). MK-801 induced amnesia for the elevated plus-maze in mice. *Behavioural Brain Research*, *131*(1-2), 221–5.

Hutter, S., Zala, S. M., & Penn, D. J. (2011). Sex recognition in zebrafish (Danio rerio). *Journal of Ethology*, *29*(1), 55–61.

Jacobs, S. a., & Tsien, J. Z. (2012). Genetic overexpression of NR2B subunit enhances social recognition memory for different strains and species. *PLoS ONE*, *7*(4).

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, *294*(5544), 1030–8.

Kanwisher, N., McDermott, J., & Chun, M. M. (1997). The fusiform face area: a module in human extrastriate cortex specialized for face perception. *The Journal of Neuroscience*, *17*(11), 4302–11.

Kappeler, P. M., Barrett, L., Blumstein, D. T., & Clutton-Brock, T. H. (2013). Constraints and flexibility in mammalian social behaviour: introduction and synthesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *368*(1618).

Keagy, J., Savard, J-F. & Borgia, G. (2009).Male satin bowerbird problem-solving ability predicts mating success. *Animal Behavior*, *78,* 809-817.

Kesslak, J. P., So, V., Choi, J., Cotman, C. W., & Gomez-Pinilla, F. (1998). Learning upregulates brain-derived neurotrophic factor messenger ribonucleic acid: a mechanism to facilitate encoding and circuit maintenance? *Behavioral Neuroscience*, *112*(4), 1012–9.

Kily, L. J. M., Cowe, Y. C. M., Hussain, O., Patel, S., McElwaine, S., Cotter, F. E., & Brennan, C. H. (2008). Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. *The Journal of Experimental Biology*, *211*(10), 1623–34.

Kogan, J. H., Frankland, P. W., & Silva, A. J. (2000). Long-Term Memory Underlying Hippocampus-Dependent Social Recognition in Mice, *56*, 47–56.

Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H., & Bonhoeffer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(19), 8856–60.

Kovalchuk, Y., Hanse, E., Kafitz, K. W., & Konnerth, A. (2002). Postsynaptic Induction of BDNF-Mediated Long-Term Potentiation. *Science (New York, N.Y.)*, *295*(5560), 1729–34.

Krause, J., Butlin, R. K., Peuhkuri, N., & Pritchard, V. L. (2000). The social organization of fish shoals: a test of the predictive power of laboratory experiments for the field. *Biological Reviews of the Cambridge Philosophical Society*, *75*(4), 477–501.

Laale, H. W. (1977). The biology and use of zebrafish, Brachydanio rerio in fisheries research.. A literature review. *Journal of Fish Biology*, *10*(2), 121–173.

Le Moal, M., Dantzer, R., Michaud, B., & Koob, G. F. (1987). Centrally injected arginine vasopressin (AVP) facilitates social memory in rats. *Neuroscience Letters*, *77*(3), 353–9.

Lee, H.-J., Caldwell, H. K., Macbeth, A. H., Tolu, S. G., & Young, W. S. (2008). A Conditional Knockout Mouse Line of the Oxytocin Receptor. *Endocrinology*, *149*(7), 3256–3263.

Lessmann, V., Gottmann, K., & Malcangio, M. (2003). Neurotrophin secretion: current facts and future prospects. *Progress in Neurobiology*, *69*(5), 341–374.

Lukas, M., Toth, I., Veenema, A. H., & Neumann, I. D. (2013). Oxytocin mediates rodent social memory within the lateral septum and the medial amygdala depending on the relevance of the social stimulus: male juvenile versus female adult conspecifics. *Psychoneuroendocrinology*, *38*(6), 916–26.

Lum, T., Huynh, G., & Heinrich, G. (2001). Brain-derived neurotrophic factor and TrkB tyrosine kinase receptor gene expression in zebrafish embryo and larva. *International Journal of Developmental Neuroscience : The Official Journal of the International Society for Developmental Neuroscience*, *19*(6), 569–87.

Lüscher, C., Nicoll, R. a, Malenka, R. C., & Muller, D. (2000). Synaptic plasticity and dynamic modulation of the postsynaptic membrane. *Nature Neuroscience*, *3*(6), 545–50.

Lynch, G., Larson, J., Kelso, S., Barrionuevo, G., & Schottler, F. (1983). Intracellular injections of EGTA block induction of hippocampal long-term potentiation. *Nature*, *305*(5936), 719–721.

Ma, Y. L., Wang, H. L., Wu, H. C., Wei, C. L., & Lee, E. H. (1998). Brain-derived neurotrophic factor antisense oligonucleotide impairs memory retention and inhibits long-term potentiation in rats. *Neuroscience*, *82*(4), 957–67.

Maaswinkel, H. (2003). Olfactory input increases visual sensitivity in zebrafish: a possible function for the terminal nerve and dopaminergic interplexiform cells. *Journal of Experimental Biology*, *206*(13), 2201–2209.

Malenka, R. C., & Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron*, *44*(1), 5–21.

Malenka, R. C., Kauer, J. A., Zucker, R. S., & Nicoll, R. A. (1988). Postsynaptic calcium is sufficient for potentiation of hippocampal synaptic transmission. *Science* , *242*(4875), 81–4.

Malenka, R. C., & Nicoll, R. A. (1999). Long-term potentiation--a decade of progress? *Science*, *285*(5435), 1870–4.

Martin, S. C., Marazzi, G., Sandell, J. H., & Heinrich, G. (1995). Five Trk receptors in the zebrafish. *Developmental Biology*, *169*(2), 745–58.

Martin, S. J., Grimwood, P. D., & Morris, R. G. M. (2000). Synaptic plasticity and Memory : An Evaluation of the Hypothesis. *Annu. Rev. Neuroscience*, 23, 649–711.

Mateo, J. M. (2004). Recognition systems and biological organization : The perception component of social recognition. *Ann. Zool. Fenni*ci, 41, 729–745.

McAllister, A. K., Lo, D. C., & Katz, L. C. (1995). Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron*, *15*(4), 791–803.

McCann, L. I., Koehn, D. J., & Kline, N. J. (1971). The effects of body size and body markings on nonpolarized schooling behavior of zebra fish (Brachydanio rerio). *The Journal of Psychology*, *79*, 71–5.

Mello, C. V, & Ribeiro, S. (1998). ZENK protein regulation by song in the brain of songbirds. *The Journal of Comparative Neurology*, *393*(4), 426–38.

Mesic, I., Guzman, Y. F., Guedea, A. L., Jovasevic, V., Corcoran, K. a, Leaderbrand, K., Radulovic, J. (2015). Double Dissociation of the Roles of Metabotropic Glutamate Receptor 5 and Oxytocin Receptor in Discrete Social Behaviors. *Neuropsychopharmacology*, 1–36.

Mizuno, M., Yamada, K., Olariu, A., Nawa, H., & Nabeshima, T. (2000). Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(18), 7116–21.

Moura, P. J., Meirelles, S. T., & Xavier, G. F. (2010). Long-term social recognition memory in adult male rats: factor analysis of the social and non-social behaviors. *Brazilian Journal of Medical and Biological Research*, *43*(7), 663–676.

Mueller, T., & Wullimann, M. F. (2009). An evolutionary interpretation of teleostean forebrain anatomy. *Brain, Behavior and Evolution*, *74*(1), 30–42.

Mueller, T., Wullimann, M. F., & Guo, S. (2008). Early teleostean basal ganglia development visualized by zebrafish Dlx2a, Lhx6, Lhx7, Tbr2 (eomesa), and GAD67 gene expression. *The Journal of Comparative Neurology*, *507*(2), 1245–57.

Murer, M. G., Yan, Q., & Raisman-Vozari, R. (2001). Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Progress in Neurobiology*, *63*(1), 71–124.

Murray, E. A., & Mishkin, M. (1998). Object recognition and location memory in monkeys with excitotoxic lesions of the amygdala and hippocampus. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *18*(16), 6568–82.

Nabavi, S., Fox, R., Proulx, C. D., Lin, J. Y., Tsien, R. Y., & Malinow, R. (2014). Engineering a memory with LTD and LTP. *Nature*.

Naimark, A., Barkai, E., Matar, M. A., Kaplan, Z., Kozlovsky, N., & Cohen, H. (2007). Upregulation of neurotrophic factors selectively in frontal cortex in response to olfactory discrimination learning. *Neural Plasticity*, 13427

Nam, R.-H., Kim, W., & Lee, C.-J. (2004). NMDA receptor-dependent long-term potentiation in the telencephalon of the zebrafish. *Neuroscience Letters*, *370*(2-3), 248–51.

Neff, B. D., & Sherman, P. W. (2003). Nestling recognition via direct cues by parental male bluegill sunfish (Lepomis macrochirus). *Animal Cognition*, *6*(2), 87–92.

Neisser, U., Boodoo, G., Bouchard, T., Boykin, A.W., Brody, N., Ceci, S.J., Halpern, D.F., Loehlin, J.C., Perloff, R., Sternberg, R.J., Urbina, S. (1996). Intelligence: knowns and unkonowns. *Am. Pychol. 51*(2), 77-101

Newman, S. W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Annals of the New York Academy of Sciences*, *877*, 242–257.

Ng, M.-C., Tang, T.-H., Ko, M.-C., Wu, Y.-J., Hsu, C.-P., Yang, Y.-L., & Lu, K.-T. (2012). Stimulation of the lateral division of the dorsal telencephalon induces synaptic plasticity in the medial division of adult zebrafish. *Neuroscience Letters*, *512*(2), 109–13.

Nicoll, R. A., Kauer, J. A., & Malenka, R. C. (1988). The current excitement in long-term potentiation. *Neuron*, *1*(2), 97–103.

Nowak, L., Bregestovski, P., Ascher, P., Herbet, A., & Prochiantz, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*, *307*(5950), 462–465.

Oliveira, R. F. (2009). Social behavior in context: Hormonal modulation of behavioral plasticity and social competence. *Integrative and Comparative Biology*, *49*(4), 423–40.

Oliveira, R. F. (2012). Social plasticity in fish: integrating mechanisms and function. *Journal of Fish Biology*.

Oliveira, R. F. (2013). Mind the fish: zebrafish as a model in cognitive social neuroscience. *Frontiers in Neural Circuits*, 17(131),1-15

OlsÉn, K., Grahn, M., Lohm, J., & Langefors, Å. (1998). MHC and kin discrimination in juvenile Arctic charr, Salvelinus alpinus (L.). *Animal Behaviour*, *56*(2), 319–327.

Pang, P. T., & Lu, B. (2004). Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. *Ageing Research Reviews*, *3*(4), 407–30.

Pastalkova, E., Serrano, P., Pinkhasova, D., Wallace, E., Fenton, A. A., & Sacktor, T. C. (2006). Storage of spatial information by the maintenance mechanism of LTP. *Science*, *313*(5790), 1141–4.

Pather, S., & Gerlai, R. (2009). Shuttle box learning in zebrafish (Danio rerio). *Behavioural Brain Research*, *196*(2), 323–7.

Patterson, S. L., Abel, T., Deuel, T. A., Martin, K. C., Rose, J. C., & Kandel, E. R. (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron*, *16*(6), 1137–45.

Perrett, D. I., Rolls, E. T., & Caan, W. (1982). Visual neurones responsive to faces in the monkey temporal cortex. *Experimental Brain Research*, *47*(3), 329–42.

Plomin, R. (2001). The genetics of g in human and mouse. *Nature Reviews Neuroscience*, *2*, 136-141.

Popik, P., Wolterink, G., De Brabander, H., & van Ree, J. M. (1991). Neuropeptides related to [Arg8]vasopressin facilitates social recognition in rats. *Physiology & Behavior*, *49*(6), 1031–5.

Portavella, M., Vargas, J. P., Torres, B., & Salas, C. (2002). The effects of telencephalic pallial lesions on spatial, temporal, and emotional learning in goldfish. *Brain Research Bulletin*, *57*(3-4), 397–399.

Reed, J. M., & Squire, L. R. (1997). Impaired recognition memory in patients with lesions limited to the hippocampal formation. *Behavioral Neuroscience*, *111*(4), 667–75.

Richter, K., Wolf, G., & Engelmann, M. (2005). Social recognition memory requires two stages of protein synthesis in mice. *Learning & Memory*, *12*(4), 407–413.

Rodríguez, F., López, J. C., Vargas, J. P., Broglio, C., Gómez, Y., & Salas, C. (2002). Spatial memory and hippocampal pallium through vertebrate evolution: Insights from reptiles and teleost fish. *Brain Research Bulletin*, *57*(3-4), 499–503.

Romero-Granados, R., Fontán-Lozano, Á., Delgado-García, J. M., & Carrión, Á. M. (2010). From learning to forgetting: Behavioral, circuitry, and molecular properties define the different functional states of the recognition memory trace. *Hippocampus*, *20*(5), 584–595.

Rosenthal, G. G., & Ryan, M. J. (2005). Assortative preferences for stripes in danios. *Animal Behaviour*, *70*(5), 1063–1066.

Schinder, A. F., Berninger, B., & Poo, M. (2000). Postsynaptic target specificity of neurotrophin-induced presynaptic potentiation. *Neuron*, *25*(1), 151–63.

Scoville, W. B., & Milner, B. (1957). LOSS OF RECENT MEMORY AFTER BILATERAL HIPPOCAMPAL LESIONS. *Journal of Neurology, Neurosurgery & Psychiatry*, *20*(1), 11–21.

Sharp, S. P., McGowan, A., Wood, M. J., & Hatchwell, B. J. (2005). Learned kin recognition cues in a social bird. *Nature*, *434*(7037), 1127–30.

Shohet, A. J. & Watt, P. J. (2009). Female guppies Poecilia reticulata prefer males that can learn fast. *Journal of Fish Biology.* 75, 1323–1330.

Sih, A., Stamps, J., Yang, L.H., McElreath, R., Ramenofsky, M. (2010). Behavior as a key component of integrative biology in a human-altered world. *Integr. Comp. Biol.* 50, 934–944.

Spence, R., Gerlach, G., Lawrence, C., & Smith, C. (2008). The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews of the Cambridge Philosophical Society*, *83*(1), 13–34.

Takayanagi, Y., Yoshida, M., Bielsky, I. F., Ross, H. E., Kawamata, M., Onaka, T., … Nishimori, K. (2005). Pervasive social deficits, but normal parturition, in oxytocin receptor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(44), 16096–101.

Thor, D. H., & Holloway, W. R. (1982). Social memory of the male laboratory rat. *Journal of Comparative and Physiological Psychology*, *96*(6), 1000–1006.

Tyler, W. J., Alonso, M., Bramham, C. R., & Pozzo-Miller, L. D. (2002). From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *9*(5), 224–37.

Van der Staay, F. J., Rutten, K., Erb, C., & Blokland, A. (2011). Effects of the cognition impairer MK-801 on learning and memory in mice and rats. *Behavioural Brain Research*, *220*(1), 215–29.

Vargas, J. P., Rodriguez, F., Lopez, J. C., Arias, J. L., & Salas, C. (2000). Spatial learning-induced increase in the argyrophilic nucleolar organizer region of dorsolateral telencephalic neurons in goldfish. *Brain Research*, *865*(1), 77–84.

Wanisch, K., Wotjak, C. T., & Engelmann, M. (2008). Long-lasting second stage of recognition memory consolidation in mice. *Behavioural Brain Research*, *186*(2), 191–196.

Ward, A. J. W., Hart, P. J. B., & Krause, J. (2004). The effects of habitat- and diet-based cues on association preferences in three-spined sticklebacks. *Behavioral Ecology*, *15*(6), 925–929.

Wersinger, S. R., Ginns, E. I., O'Carroll, A.-M., Lolait, S. J., & Young, W. S. (2002). Vasopressin V1b receptor knockout reduces aggressive behavior in male mice. *Molecular Psychiatry*, *7*(9), 975–84.

Whitlock, J. R., Heynen, A. J., Shuler, M. G., & Bear, M. F. (2006). Learning induces long-term potentiation in the hippocampus. *Science*, *313*(5790), 1093–7.

Williams, F. E., White, D., & Messer, W. S. (2002). A simple spatial alternation task for assessing memory function in zebrafish. *Behavioural Processes*, *58*(3), 125–132.

Wullimann, M. F., & Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. *J Comp Neurol*, *475*(2), 143–162.

Xu, B., Gottschalk, W., Chow, A., Wilson, R. I., Schnell, E., Zang, K., Reichardt, L. F. (2000). The role of brain-derived neurotrophic factor receptors in the mature hippocampus: modulation of long-term potentiation through a presynaptic mechanism involving TrkB. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(18), 6888–97.

Xu, X., Scott-Scheiern, T., Kempker, L., & Simons, K. (2007). Active avoidance conditioning in zebrafish (Danio rerio). *Neurobiology of Learning and Memory*, *87*(1), 72–7.

Yamada, K., & Nabeshima, T. (2003). Brain-Derived Neurotrophic Factor / TrkB Signaling in Memory Processes, *270*, 267–270.

Yamamoto, N., & Ito, H. (2000). Afferent sources to the ganglion of the terminal nerve in teleosts. *The Journal of Comparative Neurology*, *428*(2), 355–75.

Zala, S. M., & Määttänen, I. (2013). Social learning of an associative foraging task in zebrafish. *Naturwissenschaften*, 100, 469–472.

Zuberbuhler, K & Byrne, R. W. (2006). Social cognition. *Curr. Biol.*, 16, 786–790.

Zola, S. M., Squire, L. R., Teng, E., Stefanacci, L., Buffalo, E. A., & Clark, R. E. (2000). Impaired recognition memory in monkeys after damage limited to the hippocampal region. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *20*(1), 451–63.

Zou, H., Zhang, C., Xie, Q., Zhang, M., Shi, J., Jin, M., & Yu, L. (2008). Low dose MK-801 reduces social investigation in mice. *Pharmacology, Biochemistry, and Behavior*, *90*(4), 753–7.